

*Piscirickettsia salmonis; characterization and
infection in the zebrafish model*

Eva Kathrin Berger



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Eva Kathrin Berger

<http://www.duo.uio.no/>

Print Centre, University in Oslo

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Abstract

Piscirickettsia salmonis (*P. salmonis*) is a small gram-negative intracellular pathogenic bacterium found in farmed and wild salmonid and other marine fish. *P. salmonis* replicates within membrane-bound cytoplasmic vacuoles in cells of infected fish, leading to a systemic infection, known as salmonid rickettsial septicaemia (SRS), or piscirickettsiosis. Piscirickettsiosis is characterized by necrosis, pale gills, swollen kidney, and enlarged spleen, leading to high rates of mortality in infected fish populations. High rates of mortality due to *P. salmonis* infections have led to severe economic losses for the salmonid aquaculture industry, causing a loss of at least 100 million US dollars in Chile alone, and even more on a global basis. Due to the effects of piscirickettsiosis on the aquaculture, several studies have been focusing on vaccination and treatment. However, there have been few positive results due to lack of cultivation methods for *P. salmonis*, only recently it was grown independently of cultured cell lines.

In this thesis, improved procedures for growing *P. salmonis* in the laboratory were established which is a significant leap forward for research on this bacterium. Optimal medium and growth conditions to culture *P. salmonis* were obtained. Growth rate and CFU/ml could then be measured. When growth measurements were performed, it was discovered that *P. salmonis* grow better in the dark. Therefore, growth during stress conditions at different alterations in exposure to light was investigated. Several different approaches to make GFP-labeled bacteria were unsuccessful, and it was shown that *P. salmonis* have naturally occurring plasmids. Antibiotic susceptibility test was performed and *P. salmonis* strains were found to be sensitive to the majority of antibiotics used *in vitro*. Electron microscopy studies identified *P. salmonis* as pleomorphic coccus bacterium of 500-800 nm in size with the secretion of outer membrane vesicles. The outer membrane vesicles were further characterized by both scanning and transmission electron microscopy and the protein content analysis was performed by SDS-PAGE and Western blotting. Additionally, *P. salmonis* pathogenesis was studied by using the CHSE-214 cell line where the infection rate was approximately 10 % higher in the dark. Additionally, a *P. salmonis*-zebrafish embryo infection model was established. The bacteria were confirmed to be intracellular by whole-mount immunohistochemistry and by live imaging using transgenic zebrafish lines with fluorescently labeled leukocytes. This thesis lays a solid foundation for further research on *P. salmonis* and subsequent treatment against piscirickettsiosis.

Sammendrag

Piscirickettsia salmonis (*P. salmonis*) er en liten gram-negativ intracellulær patogen bakterie som har blitt funnet i oppdrett- og frittlevende arter av laksefamilien, men også i andre havfisk. *P. salmonis* kan formere seg inni cytoplasmiske vakuoler i celler hos infiserte fisk. Dette vil føre til en systemisk sykdom kjent som salmonid rikettsial septikemi (SRS), eller piscirickettsiose. Vanlige symptomer er bleke gjeller, forstørret milt, svulne nyrer og leveren er ofte gjennomsluttet av store, lyse, nekrotiske forandringer (lokal vevsdød). Dette kan føre til høy dødelighet i infiserte fiskepopulasjoner. Akvakulturindustrien som driver med oppdrett av laksefisker opplever store økonomiske tap som følge av *P. salmonis* infeksjon. Chile har et årlig økonomisk tap på minst 100 millioner amerikanske dollar, og dette tallet blir enda større på en global basis. Disse store økonomiske tapene forårsaket av piscirickettsiose i akvakulturen har ført til studier av vaksiner og behandling. Dette har gitt få positive resultater, mest sannsynlig på grunn av manglende kultiverings metoder for *P. salmonis*. Det ble lenge antatt at *P. salmonis* kun kunne vokse i fiskecellelinjer, men nylig ble det for første gang påvist at den kan bli kultivert uavhengig av cellelinjer.

I denne oppgaven ble det etablert forbedrede prosedyrer for å dyrke *P. salmonis* på laboratoriet. Dette er et stort framskritt som vil gjøre forskningen på denne bakterien enklere. Det optimale mediet og forholdene for dyrkning ble funnet, og vekst og CFU/ml ble målt. Under vekst-målingene ble det observert at *P. salmonis* vokste bedre i mørket enn i lyset. Derfor ble bakterien utsatt for stress i tillegg til lys og mørke forhold. Det ble testet flere metoder for å lage GFP-merkede *P. salmonis* mutanter, uten noe suksess. I denne oppgaven ble det også vist at *P. salmonis* har naturlige plasmider. I tillegg ble antibiotika sensitivitet testet som viste at bakteriene var veldig følsomme for ulike typer antibiotika *in vitro*. Elektron mikroskop ble videre brukt for å identifisere størrelsen og formen på bakteriene som viste at de var runde med en størrelse på 500-800 nm. Ved å bruke skanning og transmisjon elektronmikroskop ble det observert at *P. salmonis* sekreter ytermembranvesikler. Protein innholdet i disse vesiklene ble videre analysert ved SDS-PAGE og Western blotting. *P. salmonis* patogenese ble også studert ved å benytte CHSE-214 cellelinjen der infeksjonen viste seg å være 10 % høyere i mørke, og en *P. salmonis*-sebrafisk embryo infeksjonsmodellen ble etablert. Bakterien ble bekreftet å være intracellulær med en immunhistokjemisk metode og ved å bruke transgene sebrafisklinjer med fluoriserende leukocytter. Resultatene i denne oppgaven gir et godt fundament for forskning på *P. salmonis* og for videre studier nødvendig for å finne en effektiv behandling imot piscirickettsiose.

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Abbreviations

µg	Microgram
µl	Microliter
µM	Micromolar
BLAST	Basic local alignment tool
β-Me	β-Mercaptoethanol
Calcein AM	Acetoxymethyl diacetylester of calcein
CFU	Colony forming units
CHSE-214	Chinook salmon embryo-214 cells
CPE	Cytopathic effect
CT	Cycle threshold
DAPI	4', 6-diamidino-2-phenylindole
dH ₂ O	Distilled water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dpi	Days post infection
EBFC	Eugon broth with FeCl ₃ and Casamino Acids
ECA	Eugon Chocolate Agar
FAO	Fisheries and Aquaculture Department
FBS	Fetal bovine serum
hpf	Hours post fertilization
LPS	Lipopolysaccharide
ml	Milliliter
mM	Millimolar

MQ water	Milli-Q water
NVI	Norwegian Veterinary Institute
OD	Optical density
OMVs	Outer membrane vesicles
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PTU	1-phenyl-2-thiourea
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
rpm	Revolution per minute
RT-qPCR	Real time quantitative polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SRS	Salmonid rickettsial septicaemia
SW	Seawater
TCA	Trichloroacetic acid
TEM	Transmission electron microscopy
Tricaine	Tricaine methanesulfonate MS-222
Triton X-100	Polyethylene glycol p-(1.1.3.3-tetramethylbutyl)-phenyl ether
Tween 20	Polyoxyethylene (20) sorbitan monooleate
UA	Uranyl acetate
WGA	Wheat germ agglutinin

1 Introduction

1.1 Aquaculture

According to the Fisheries and Aquaculture department of Food and Agriculture Organization of the United Nations (FAO), aquaculture is the farming of aquatic organisms including molluscs, crustaceans, aquatic plants and fish. Farming of fish is the most common form of aquaculture. The global aquaculture production of farmed fish in 2012 was approximately 66 million tonnes, accounting for around 50 % of the world's fish food supply (Bostock et al., 2010). Asia dominates this production, with China as the largest producer. However, as a market for fish and seafood; Europe and North America are not far behind, and the production is still increasing (Bostock et al., 2010). As the human population continues to expand its reliance on farmed fish production as an important source of protein will also increase (Naylor et al., 2000). Thus the production of farmed fish will need to become even higher during the next years. According to the latest estimates (Food and Agriculture Organization of the United Nations, 2014a), fish farms would have to produce 50 million additional tonnes of food fish by 2030 to meet the increasing consumption. In addition, there is a common opinion that this increased production will relieve pressure on ocean fisheries, but in fact the opposite is true for some types of aquaculture (Naylor et al., 2000). Farming carnivorous species like salmon and shrimp requires large inputs of wild fish for feed. This has led to the use of industrial fishing to support aquaculture. For instance, from 1985 to 1995, the world's shrimp farmers used approximately 36 million tons of wild fish to produce just 7.2 million tons of shrimp (Frankic and Hershner, 2003). The wild fish that often are used as feed are anchovies, herring, sardines and mackerel, which also can be used as food for human consumption. From 1992 to 2003 total fishmeal and fish oil used in salmon production have increased from 261 to 982 thousand tonnes (Tacon, 2005). Aquaculture is a possible solution for the world's need for food, however it is also a contributing factor to the collapse of fisheries stocks worldwide (Naylor et al., 2000).

Several countries including Norway have made large investments in the aquaculture industry, mainly due to the fact that it is a profitable business and Norway's long coastline surrounded by cold, fresh seawaters provides excellent conditions for aquaculture industry. Norway has a long history in the fish industry and the aquaculture began in the early 1970s, and has

expanded ever since. Today, fish is Norway's third most valuable export product, with a total aquaculture production of more than 1.3 million tonnes in 2012. The primary farmed fish species are the salmonid fish, such as Atlantic salmon *Salmo salar* and Rainbow trout *Oncorhynchus mykiss* (Food and Agriculture Organization of the United Nations, 2014b) with the sale of salmon in 2012 came to a first-hand value of 30 billion Norwegian kroner according to The Central Bureau of Statistics (Fig. 1.1.1) (Statistics Norway, 2014).

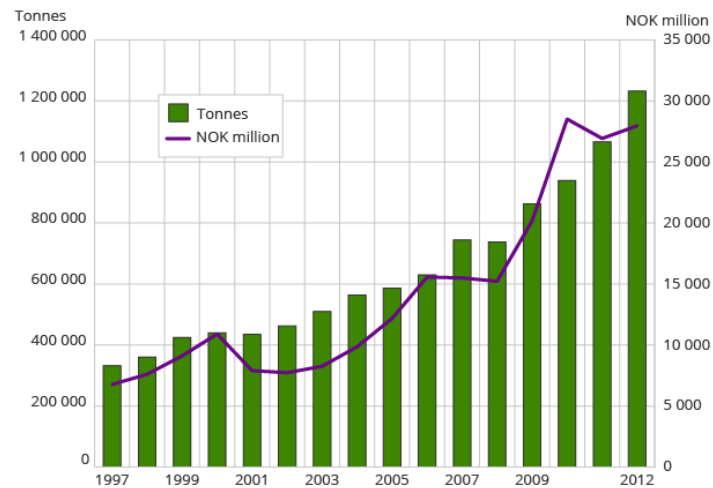


Fig. 1.1.1: Amount of salmon produced in tonnes from year 1997 to 2012 is indicated by the green bars. The purple line shows value in million NOK (Statistics Norway).

An important factor allowing further increase in fish production is the need to better control the many viral and bacterial diseases that cause significant losses in aquaculture such as the parasitic sea lice. Sea lice are the most damaging parasite to the salmonid farming industry (Costello, 2009). Exposure to viral and bacterial pathogens is impossible to avoid for species reared in nets in open aquatic environment (Sommerset et al., 2005). The annual economic loss associated with fish pathogens between 1990 and 1992 in China was estimated to more than 120 million US dollars (Pridgeon and Klesius, 2013). The same is the case for the Chilean salmon aquaculture industry, where loss due to diseases caused by fish pathogens exceeds 100 million US dollars annually (Bustos et al., 2006). The bacterial diseases alone affecting aquaculture production collectively lead to global losses of hundreds of millions to billions of dollars annually (Pridgeon and Klesius, 2013). Many viruses e.g infectious salmon anemia virus cause serious diseases in farmed Atlantic salmon (*Salmo salar*) (Crane and Hyatt, 2011). Also several bacterial species, such as *Aeromonas*, *Piscirickettsia*, *Vibrio* and *Moritella* (Toranzo et al., 2005) are pathogenic for fish.

Several antibiotics are used for the treatment of fish bacterial diseases (Samuelsen et al., 2006). However, treatment with antibiotics is limited by several practical constraints. Antibiotics can be administered by injections, bath treatment or orally. Injections are expensive and stressful for the fish, and in bath treatments the salt content in seawater can reduce the ability of adult fish to absorb the antibiotics. Oral administration with food pellets is the preferred method for adult fish, however this treatment is intended to control the level of infection in the group rather than infection in individual fish (Samuelsen et al., 2006). This is due to loss of appetite of infected fish and therefore oral treatment will mainly be delivered to uninfected fish. In addition, overuse of antibiotics in the aquaculture can lead to antibiotic resistant pathogens. Therefore, the key point in the successful fish production is the development of vaccines. At present, there are many vaccines on the market against some of the most serious bacterial diseases that cause major problems for the aquaculture. For example injectable vaccines were developed in 1990s against furunculosis caused by the bacterium *Aeromonas salmonicida* (Sommerset et al., 2005). After a few years of testing with different vaccine adjuvants and antigen combinations, it became evident that all antigens in one oil-adjuvanted vaccine was the product of choice, and the excellent efficacy of these vaccines reduced the use of antibiotics (Sommerset et al., 2005). There are several vaccines developed against bacterial infections such as cold-water vibriosis or “Hitra disease” caused by *Vibrio salmonicida* (Lillehaug, 1990). However, both vaccines and antibiotics have limited effect if the pathogen has an intracellular nature, as can be exemplified by *Piscirickettsia salmonis* (*P. salmonis*). *P. salmonis* infection is one of the most serious problems in the Chilean aquaculture industry (Sommerset et al., 2005), which also affects Norwegian fish production to some extent. After the outbreak of piscirickettsiosis in Norway in 1987 (Olsen et al., 1997), only sporadic outbreaks have been recorded.

P. salmonis has not been extensively studied, which hampers the development of therapeutic strategies. In 1989, approximately 1.5 million Coho salmon (*Oncorhynchus kisutch*) died from the infection in Chile, this caused an economic loss of 10 million US dollars (Cvitanich et al., 1991). Since the original outbreak in 1989, the number and severity of outbreaks have since increased (Rozas and Enríquez, 2013). The disease is a threat to the salmonid industry, not only for marine net pens, but also freshwater hatcheries (Gaggero et al., 1995). According to the National Service of Fisheries and Aquaculture, there were 664 fish diagnostics positive to piscirickettsiosis in the first nine months of 2012 (Pèrez, 2012). In addition, each new outbreak shows a different character with increased bacterial virulence; thereby introducing

even more challenges with regards to diagnosis, prevention, treatment and control (Rozas and Enríquez, 2013).

1.2 *Piscirickettsia salmonis*

1.2.1 Classification and properties

The bacterium *P. salmonis* was initially grouped in the family Rickettsiaceae and was referred to as rickettsia-like, because of the similarities with other bacteria in the family (Cvitanich et al., 1991, Fryer et al., 1990). Subsequently, the bacterium was classified in a new family *Piscirickettsiaceae* in the phylum Proteobacteria, class Gammaproteobacteria, order Thiotrichales (Fryer and Hedrick, 2003) and assigned to a new genus and species *Piscirickettsia salmonis* (Fryer et al., 1992). This was due to molecular phylogenetic methods based on sequencing of the 16S rRNA gene. The sequence of the 16S rRNA gene confirmed the association of *P. salmonis* with the Gammaproteobacteria, instead of the Rickettsia, that are grouped within the Alphaproteobacteria (Fryer and Hedrick, 2003).

P. salmonis is a small, fastidious, gram-negative intracellular pathogenic bacterium found in farmed and wild salmon and other marine fish. The bacterium is generally described as non-motile, non-encapsulated, aerobic, pleomorphic, predominately coccoid with an approximate diameter of 0.5-1.5 µm, but can also be found in pairs or ring-shaped structures (Fryer et al., 1990, Fryer et al., 1992). *P. salmonis* replicates by binary fission within membrane-bound cytoplasmic vacuoles in cells of infected fish or fish cell lines, for instance Chinook salmon embryo-214 cell line (CHSE-214) (Fryer et al., 1990). Previously, *P. salmonis* were considered unable to grow outside a host cell (Fryer et al., 1990), but recently several publications describe media in which the bacterium is able to grow, both on agar medium and in liquid culture (Mikalsen et al., 2008, Mauel et al., 2008, Gómez et al., 2009, Yanez et al., 2013, Henriquez et al., 2013). Mikalsen et al. (2008) described an agar culture medium based on cysteine heart agar supplemented with 5 % ovine blood (CHAB), and after 6 days of incubation *P. salmonis* colonies had reached 1 mm in diameter. On the other hand, Yanez et al. (2013) described two novel blood-free solid media; tryptone soy with ferric nitrate (Austral-TSFe) agar and tryptone soy with heamoglobin (Austral-TSHem) agar. The growth of *P. salmonis* was approximately 8-10 days on the Austral-TSHem and Austral-TSFe plates, which was a significant leap forward, as blood-free plates is preferred, since blood can be a

cause of contamination or difficult to obtain. Henriquez et al. (2013) has also demonstrated a blood-free bacteriological liquid medium with yeast extract and peptone. It was reported that *P. salmonis* could grow to an OD₆₀₀ value of 1.7 in only 37.5 hours. The ability to grow *P. salmonis* in laboratory conditions allows one to study its growth and pathogenesis. This may lead to a possible vaccine development, providing a treatment plan for the disease caused by *P. salmonis*, known as salmonid rickettsial septicaemia (SRS), or piscirickettsiosis (Mauel et al., 2008).

1.2.2 Piscirickettsiosis and current state of treatment strategy

In the late 1980s piscirickettsiosis started to spread among net pen-reared salmonid stocks in the aquaculture industry in Chile. It appeared that the disease began after fish were introduced into the seawater net pens (Bravo and Campos, 1989). Intensive efforts were made to classify the organism responsible for the disease, and eventually the bacterium was classified as *Piscirickettsia salmonis* and the disease was then named piscirickettsiosis, or SRS (Fryer et al., 1992). Coho salmon (*Oncorhynchus kisutch*) was the main species affected by the disease with a mortality rate of 30 - 90 % in 1989 (Bravo and Campos, 1989). The disease was, however, not restricted to Chile and in later years piscirickettsiosis has also been observed in Ireland (Rodger and Drinan, 1993), Norway (Olsen et al., 1997) and Canada (Cusack et al., 2002), where it affected not only Coho salmon, but also other farmed and wild salmonid and marine fish (Mikalsen et al., 2008). Reports of *P. salmonis* infection in marine fish include European seabass, *Dicentrarchus labrax* (McCarthy et al., 2005) and white seabass *Atractoscion nobilis* (Arkush et al., 2005). Numerous anti-microbial agents have also been used to control the disease without any reasonable success.

The route of disease transmission is not completely understood, but it has, however, been demonstrated that *P. salmonis*'s main entry site is through the skin and gills (Smith et al., 1999). In the study performed by Smith et al. (1999), Rainbow trout were infected with *P. salmonis* at 6 different entry sites to study the routes of natural infection. *P. salmonis* was reported to penetrate the skin in the absence of injury and without the assistance of another vector. In addition, *P. salmonis* could reach the gill capillaries and disseminate through the body in a relative short time. However, oral exposure is not an important route of transmission, and the theory is that the low pH and digestive enzymes in the stomach inactivates the bacterium. Similar studies has been performed in Atlantic salmon, where they

showed that gills might be important portals of entry for natural transmission of *P. salmonis* (Almendras et al., 1997).

No reservoirs or vectors are known, but there are many parasitic crustaceans in the marine environment that could serve as vectors for *P. salmonis* (Fryer et al., 1990). *P. salmonis* is obviously protected from desiccation in aquatic environments, and therefore it is possible that no vector is required (Fryer et al., 1990). However, it has been shown that *P. salmonis* can replicate in insect- and frog-derived cell lines (Birkbeck et al., 2004a), and a parasitic isopod associated with farmed salmon in Chile, was identified as a host for *P. salmonis*. Subsequently, a reservoir in marine finfish species has been suspected, but not proven (Rozas and Enríquez, 2013). In addition, *P. salmonis* are not only restricted to salmonid hosts, as the disease has been observed also in non-salmonids (McCarthy et al., 2005). Therefore, it is some evidence that a vector and a reservoir may exist, but further studies are needed.

The experimental work on whether vertical transmission occurs is in progress, yet little is known. *P. salmonis* have been described in the ovaries, testicles and coelomic fluid of infected salmon (Cvitanich et al., 1991). Larenas et al. (2003), observed that fish eggs were infected from an early development phase in ovary tissue and that the tissue could produce gametes that were viable carriers of the bacteria (Larenas et al., 2003). Horizontal, or fish-to-fish dependent transmission of *P. salmonis* has been demonstrated in both fresh and salt water, and it is enhanced by direct contact among fish (Almendras et al., 1997). Subsequently, natural horizontal transmission in seawater was demonstrated in stocks of salmon. After *P. salmonis* introduction into the fish bath, mortality occurred within 2 weeks (Bravo, 1994). The same was observed by Cvitanich et al. (1991); horizontal transmission can occur in Coho salmon held in seawater or fresh water without parasite vectors (Cvitanich et al., 1991). Another hypothesis is that *P. salmonis* can be released through fish feces, and survive to infect other fish (Cvitanich et al., 1991). *In vitro* experiments examining extracellular survival of *P. salmonis* from Coho salmon showed no detection of infectious *P. salmonis* after exposure to freshwater, but *P. salmonis* was detected in salt water (Lannan and Fryer, 1994). Due to this finding, horizontal transmission in marine waters is very probable. However the situation is not the same in fresh water where it seems like the bacterium has to be protected within host cells or a vector if horizontal transmission is to occur (Lannan and Fryer, 1994). *P. salmonis* can survive for up to 14 days in seawater (Lannan and Fryer, 1994), and the long survival of *P. salmonis* in seawater, is possibly due to the ability to create a biofilm and thus

withstand stress conditions (Marshall et al., 2012). Biofilms are a collection of microbial cells that are densely packed, can adhere to solid surfaces and surround themselves with secreted polymers (Costerton et al., 1999). Environmental conditions can trigger the transition from free living to a life on a surface, or in cell aggregates (Marshall et al., 2012). Many pathogenic bacteria produce biofilms as an important survival strategy and biofilm formation may promote survival of *P. salmonis* in seawater, and thereby allow for the infection of fish.

The fish that are affected by *P. salmonis* often exhibit symptoms, though the clinical signs are variable. Severely affected fish can appear darker in color, with pale gills, are lethargic, loss of appetite, show skin lesions or ulcers and have abnormal swimming behavior, such as swimming near the surface or edges of the cages. In addition, most affected fish have internal symptoms where several organs and tissue are involved, like swollen kidney, liver and spleen (Cvitanich et al., 1991, Fryer and Hedrick, 2003) (Fig. 1.2.1).



Fig. 1.2.1: Symptoms associated with piscirickettsiosis in salmon species. A: Skin ulcers at different areas of the body. B: Big skin ulcer. C: Pale liver (Rozas and Enríquez, 2013).

Multifocal necrosis can also happen in the more acute phase of piscirickettsiosis, and it is usually followed by granulomatous inflammation. A granuloma is a collection of immune cells, such as macrophages, and it may occur in many diseases. Granulomas are usually formed as a result of hypersensitivity responses or of the persistence of a non-degradable product (Mukhopadhyay et al., 2012). Macrophages are phagocytic cells, and after ingestion, the pathogen will be located in a membrane-bound vacuole (phagosome). However, the trafficking of this vacuole and the bacterial survival strategies vary considerably (Garcia-del Portillo and Finlay, 1995). To succeed as an infectious pathogen, the pathogen must have a survival strategy. It has to colonize a host, reach a niche, avoid host defense, replicate, exit from the host and be transmitted to a new host (Rojas et al., 2013). The bacteria without an

intracellular survival strategy are digested, due to phagosome fusion with the lysosomal compartment. This compartment contains hydrolytic enzymes and a low pH, that can facilitate killing of the pathogens, and in some cases also the presentations of antigens to the immune system (Ernst et al., 1999). There are several different strategies to avoid the hostile environment of the macrophage phagosome (Fig. 1.2.2). The pathogen can reside in a phagosome that blocks the fusion with lysosomes, as seen in *Legionella* and *Mycobacterium* (Ernst et al., 1999). They can escape the phagosome after lysing the phagosomal membrane and resides freely in the host cell cytoplasm, as seen in *Listeria* and *Shigella* infection (Ernst et al., 1999). *Salmonella* species have another strategy; they have adapted to resist the antimicrobial activity of the fused phagolysosome (Ernst et al., 1999). However, which escape strategy is used by the pathogen *P. salmonis* is still unknown.

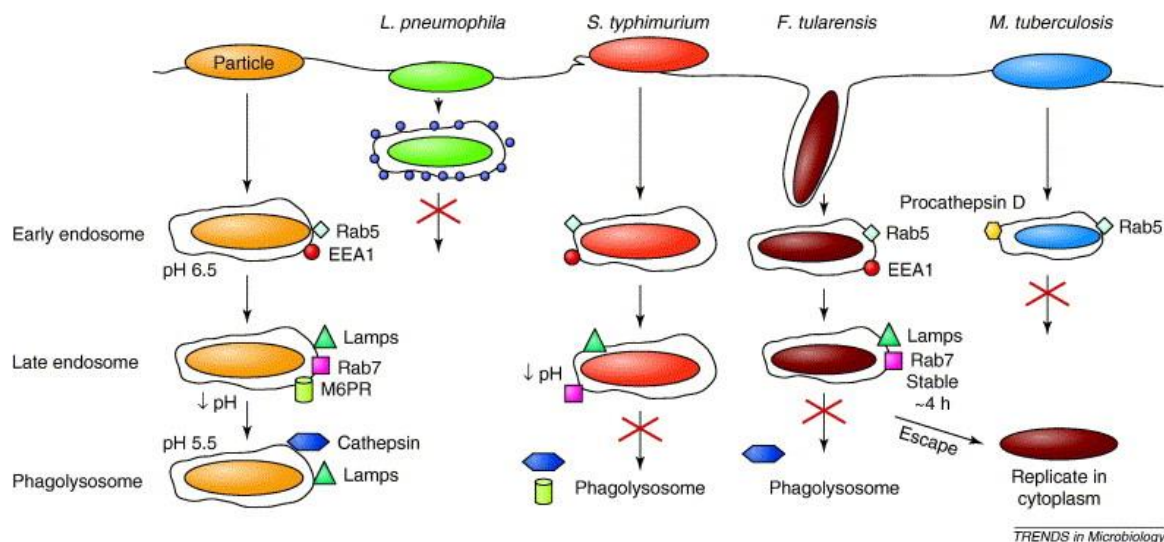


Fig. 1.2.2: Trafficking within the endocytic pathway and the different intracellular pathogens have evolved various ways of avoiding being degraded within the cell (Santic et al., 2006).

In *P. salmonis* infection, macrophages can be found within areas of necrosis, where they harbor intracellular aggregates of *P. salmonis*. According to Rojas et al. (2009) this obligate intracellular pathogen can survive within phagocytic cells. The bacterium is able to survive and propagate inside salmonid macrophages and monocyte-like cells without inducing cytopathic effect (CPE) (Rojas et al., 2009, McCarthy et al., 2008). This is a potential survival strategy, and the combination of intracellular growth and regulation of macrophage cell death could be the basis for *P. salmonis*'s pathogenesis. Subsequently, it has been shown that *P. salmonis* can induce apoptosis (programmed cell death) in macrophages and monocyte-like cells (Rojas et al., 2010). The mechanism behind this process has not been described, but it

could be that *P. salmonis* produces toxins that activate macrophage apoptosis, but no such toxins have been identified yet (Rojas et al., 2010). The virulence factors of this pathogen are poorly known though it has been recently found that *P. salmonis* secretes extracellular products, and the hypothesis is that some of these compounds may have a cytotoxic effect *in vitro* and probably mediate some tissue damage *in vivo* (Rojas et al., 2013). Recently, the first draft genome sequence of *P. salmonis* LF-89 was published (Eppinger et al., 2013) which can be important for revealing *P. salmonis* virulence factors. The 3,388,517-Mbp draft genome sequence had a G+C content of 39.2%. Eppinger et al. (2013) found the presence of type IV pilus genes and bacteriophage genes in the genome. Type IV secretion genes were found in large clusters, which may be critical for intracellular survival. Finally, flagellar and chemotaxis genes were present in the genome, which is unexpected due to the fact that the bacterium is characterized as non-motile. The flagellar gene organization suggests that *P. salmonis* synthesizes a single polar flagellum (Eppinger et al., 2013). Further mapping of the *P. salmonis* genome may provide new information in regards to this pathogen, which still is a major problem in aquaculture.

Piscirickettsiosis has led to severe economic losses for the Chilean salmon aquaculture industry, causing a loss of at least 100 million US dollars in Chile alone and even more on global basis. Today, piscirickettsiosis is still a big threat to brackish and seawater aquaculture (Bustos et al., 2006). A reason for this threat is the difficulties in using effective antibiotics and developing good vaccines, due to the bacterium's intracellular nature (Carryn et al., 2003). Even though *P. salmonis* is sensitive to a variety of antibiotics *in vitro* (Mikalsen et al., 2008), the situation is not the same *in vivo*, presumably due to insufficient concentration of antibiotics within the host. Still treatment with antimicrobial drugs is the main therapy against piscirickettsiosis (Cvitanich et al., 1991). There are only four antibiotics in the Norwegian aquaculture that are used to treat fish pathogens: florfenicol (only for salmon), oxolinic acid (salmonids) and sulphadiazin/trimethoprim (finfish) (Norwegian Medicines Agency, 2014). In regard to *P. salmonis* infection, orally administered oxolinic acid is the drug of choice, even though the response is slow and it has to be given repeatedly (Fryer and Hedrick, 2003). A lot of the antibiotics tested *in vitro* cannot be used in aquaculture because they are used to treat human diseases. According to the Center of food safety, the use of antibiotics may lead to bacterial resistance which will undermine the effectiveness of those antibiotics in treating human illnesses.

Using antibiotics can cause a number of side effects such as development of resistant bacterial strains and pollution of the environment. Antibiotics can be administrated in fish cultures by immersion baths, feed or injections. However, since there are several disadvantages with the delivery of antibiotics to the fish, finding an effective vaccine for piscirickettsiosis is preferable, but only a small number of vaccine trials have been reported. This does not mean that they do not exist, but are rather undisclosed by the private actors. Attempts to use whole-cell bacterins have shown variable results (Kuzyk et al., 2001b, Smith, 1995). In addition, vaccines trials based on formalin-inactivated and heat-inactivated cells have been established (Birkbeck et al., 2004b). However, better protection was observed when a recombinant vaccine was established (Kuzyk et al., 2001a), and it did not take long before the next vaccine based on a mixture of recombinant proteins was introduced into the market (Wilhelm et al., 2006). At present, there are 33 commercially injectable vaccines against piscirickettsiosis available in Chile, where 29 are inactivated vaccines and 4 are subunit vaccines. In total, 2 of these vaccines are oral and 31 are injectable (Rozas and Enríquez, 2013). The vaccines have variable long-term efficacy, but at least they protect the fish after transfer of fish from fresh water to seawater. After this the fish are susceptible to a second, more aggressive form of piscirickettsiosis, which has proved to be much more difficult to protect the fish against (Tobar et al., 2011).

To find a therapy against piscirickettsiosis, more research is needed both on the pathogenesis and within vaccine development. A possible pathogenesis factor that might be important for *P. salmonis* is the outer membrane vesicles (OMVs), which are naturally produced by gram-negative bacteria (Collins, 2011). These vesicles have previously been used successfully as vaccines (Park et al., 2011) and have shown to give protective immunity against other fish pathogens, including *Francisella noatunensis* (Brudal, 2014). The interest in the OMVs as vaccines has expanded, and it is possible that they can be used as a vaccine against piscirickettsiosis in the future. However, to further study potential vaccine candidates against *P. salmonis*, further studies of the pathogenesis is needed, and this might be provided by the establishment of more advanced infection models, like the extensively studied zebrafish embryo model.

1.3 Zebrafish as a model system

The zebrafish (*Danio rerio*) is a small tropical fresh-water fish that is popular in aquariums. They are usually characterized by its white and dark stripes, and can reach a size up to 5 cm in length as adults. The zebrafish has been known since 1822, but in 1960 it was discovered as an excellent model for research (Sullivan and Kim, 2008). The fish offers several advantages as an animal model: they are one of the smallest vertebrate animals with a fully developed adaptive and innate immune system, require minimal laboratory space, they have short generation time- a single female can produce up to 200-300 embryos per mating, easy maintenance, low cost, fully sequenced genome, availability of transgenic lines (Fig. 1.3.1) and molecular research tools. Interestingly, the innate immune system is functional early in zebrafish maturation, with macrophages and neutrophils active by 48 hours post fertilization (Allen and Neely, 2010). The adaptive immune system on the other hand is not fully developed for another 4-6 weeks (Lam et al., 2004). This enables an opportunity to study the innate immune system without the interference of the adaptive immune system.

Furthermore, embryos develop externally and- due to their transparency at the embryo- and early larval stage, many processes can be visualized using light microscopy (Meeker and Trede, 2008). Some mutant strains maintain their transparency throughout life (White et al., 2008). Using these fish lines in combination with fluorescently labeled pathogens allows real-time *in vivo* observations of disease progression (Phelps and Neely, 2005). Moreover, fish lines are established that possesses fluorescently labeled immune cells, like macrophages or neutrophils. Here, fluorescent labeling can provide essential information about early stages of infection and the interaction between the pathogen and the immune cells of the fish.



Fig. 1.3.1: Adult zebrafish, wild type strain AB (upper left corner) and some of the different transgenic lines (White et al., 2008). The wild type strain AB has three distinct classes of pigment cells arranged in stripes: black melanophores, reflective iridophores and yellow xanthophores. The nacre strains lack the melanocytes, while the roy strains lack the iridophores. Finally, the casper strains complete lack of all melanocytes and iridophores.

There are only few things that are not fully accomplished in the zebrafish model. These are the lack of antibodies against zebrafish proteins and difficulties in establishment of isolated cell cultures (Meeker and Trede, 2008). Remarkably, the zebrafish immune system is quite similar to humans and other vertebrates, which makes it a versatile model (Howe et al., 2013). The zebrafish has also been proven to be a robust model for studying pathogenesis, host-pathogen interactions at several stages of the infection; screening, drugs and vaccines. To exemplify, the zebrafish has been used to study intracellular pathogens, such as *Mycobacterium marinum* (Swaim et al., 2006) an extensively used model for human *Mycobacterium tuberculosis* infections and *Francisella* (Brudal et al., 2014), which has provided important information in regard to their pathogenesis.

2 Aims of the study

P. salmonis was only recently, for the first time, grown independent of cell lines. Thus, little is still known about *P. salmonis* growth and pathogenesis.

The main goal of this Master's thesis was therefore to:

1. Establish growth of the different *P. salmonis* strains in the laboratory.
2. Make fluorescently-labeled GFP mutants strains.
3. Establish a *P. salmonis*-zebrafish embryo infection model.
4. Treat piscirickettsiosis by nanoparticle encapsulation of antibiotics.

This will be a starting platform for research on *P. salmonis* and subsequent treatment against piscirickettsiosis.

3 Materials and methods

3.1 Bacterial strains, media and growth conditions

The three isolates of *P. salmonis* used in this study was a kind gift from Duncan J. Colquhoun (Oslo, NVI) (Table 3.1.1). These bacteria were routinely cultured at 20°C on Eugon Chocolate Agar (ECA), containing 30.4 g/L BD Bacto™ Eugon Broth (Becton, Dickinson and Company, Franklin lakes, NJ, USA), 15 g/L Agar Bacteriological (Thermo Fisher Scientific, Hudson, NH, USA) and 5% bovine blood (Håttunlab AB) or in liquid culture (EBFC) with BD Bacto™ Eugon Broth supplemented with 2 mM FeCl₃ (Sigma-Aldrich Co., St. Louis, MO, USA) and 1% Casamino Acids (BD). Bacteria were kept at -80°C for long term storage in autoclaved 10% skimmed milk (BD Difco) or in BD Bacto™ Eugon Broth supplemented with 20% glycerol (Sigma-Aldrich). Additionally, *E.coli* was routinely cultured at 37 °C on Luria Agar (LA-plates) and in Luria Broth (LB-media).

Table 3.1.1: Bacterial strains used in this study.

Isolate	Species isolated from	Country
<i>P. salmonis</i>		
<i>P. salmonis</i> NVI 5692	Atlantic salmon (<i>Salmo salar</i>)	Norway
<i>P. salmonis</i> NVI 5892	Atlantic salmon (<i>Salmo salar</i>)	Canada
<i>P. salmonis</i> 5896	Unknown	Unknown
<i>E.coli</i>		
<i>E.coli</i> S17.1		

3.2 Identification of *P. salmonis* by 16S rRNA sequencing

The strains used in this study were identified as *P. salmonis* by 16S rRNA sequencing. Boiling preparation was made of *P. salmonis* 5692, 5892 and 5896 and a nested polymerase chain reaction (PCR) amplification was carried out. Primers used in this study are given in more detail in Table 3.2.1. In the first round of amplification the bacterial 16S primers, EubA and EubB (GIBCO®, Invitrogen, Paisley, UK) were used and *P. salmonis* specific primers, PS2S and PS2AS from the same manufacturer were used in the second round. The PCR reaction was set up using Dynazyme screening master mix which contained Dynazyme II DNA polymerase (Thermo scientific), and 2 µl template DNA from each strain were used in

the reaction. In addition, boiling preparation of *E.coli* S17.1 was used as a control and negative controls were made without any template DNA. The mixture was denatured at 95°C for 5 minutes, amplified with 35 cycles of 94°C for 30 seconds, 50°C for 40 seconds, 72°C for 40 seconds and finally the last extension step of 72°C for 5 minutes in a GeneAmp PCR system 2700- PCR machine (Applied Biosystems, Foster City, CA, USA.). For the second round of amplification, 2 µl of the first round of reaction was used as a template. The mixture was denatured at 95°C for 5 minutes, amplified with 35 cycles of 94°C for 30 seconds, 61°C for 40 seconds, 72°C for 40 seconds and finally the last extension step of 72°C for 5 minutes. All samples were examined for specificity on a 1% agarose gel (Sigma-Aldrich) and the gel products were cleaned from the gel by following E.Z.N.A gel extraction kit (Omega Bio-Tek, Norcross, GA) (see appendix B: Kit protocols). DNA concentration and purity were measured with a Picodrop spectrophotometer (Picodrop Ltd, Cambridge, UK) and the products were then sent to sequencing (ABI-lab, IBV, UiO).

Table 3.2.1: Primers used in 16S rRNA sequencing.

Primer	Sequence	Specificity
EubB	5'-AGAGTTTGATCMTGGCTCAG-3'	Eubacterial
EubA	5'-AAGGAGGTGATCCANCCRCA-3'	Eubacterial
PS2S	5'-CTAGGAGATGAGCCCGCGTTG-3'	<i>P. salmonis</i> 16S
PS2AS	5'-GCTACACCTGCCAAACCACTT-3'	<i>P. salmonis</i> 16S

3.3 Antibiotic susceptibility

P. salmonis 5692, 5892 or 5896 was grown in Eugon broth supplemented with 2mM FeCl₃ and 1 % Casamino Acids. Each strain was plated evenly on two ECA plates (3 ml per plate) and excess of liquid was removed. The bacterial culture on the plates was allowed to solidify on the bench and different antibiotic tablets, with 6 tablets per plate (Table 3.3.1), were placed on the plates. Growth was observed after one week incubation at 20 °C and a zone of sensitivity was measured around each tablet.

Table 3.3.1: Antibiotic tablets used to test antibiotic susceptibility by a disc-diffusion method.

Abbreviations	Antibiotic	Tablet concentration (µg)	Manufacturer
G-25	Sulfisoxazole	250 µg	BD
C-30	Chloramphenicol	30 µg	BD
Mec-10	Mecillinam	10 µg	BD
GM-30	Gentamycin	30 µg	BD

SXT	Sulfamethoxazole/trimethoprim	23.75 µg/1.25 µg	BD
Ox-5	Oxacillin	5 µg	BD
Te-30	Tetracyclin	30 µg	BD
Cip-5	Ciprofloxacin	5 µg	BD
Am-10	Ampicillin	10 µg	BD
SD-25	Sulfadiazine	250 µg	BD
CN-30	Cephalexin	30 µg	BD
SMX	Sulfamethoxazole	23.75 µg	BD
Rif-5	Rifampicin	5 µg	BD

3.4 Growth curves

P. salmonis 5692, 5892 or 5896 was cultured in 10 ml of liquid media and incubated at 20 °C with gentle shaking (100 rpm) in an Innova 4230 incubator and shaker (New Brunswick Scientific, Edison, NJ, USA). Optical density (OD) at 600 nm was measured three times a day with a Bio Photometer (Eppendorf AG, Hamburg, Germany). The optimal OD₆₀₀ of the inoculum was determined by diluting the culture at different ODs in 50 ml medium. The determined optimal inoculum was then used further for growth curves measurements of each strain. Samples were taken out from the liquid culture every second day and plated evenly on an ECA plates to check for contaminations.

Growth of *P. salmonis* was in addition tested after starvation in autoclaved seawater and during alterations in access to light to further evaluate the effects of different conditions on growth in liquid medium. The cultures were incubated at 20 °C, and when they reached an appropriate OD₆₀₀, the cultures were diluted and incubated at the various growth conditions, as described in Fig. 3.4.1.

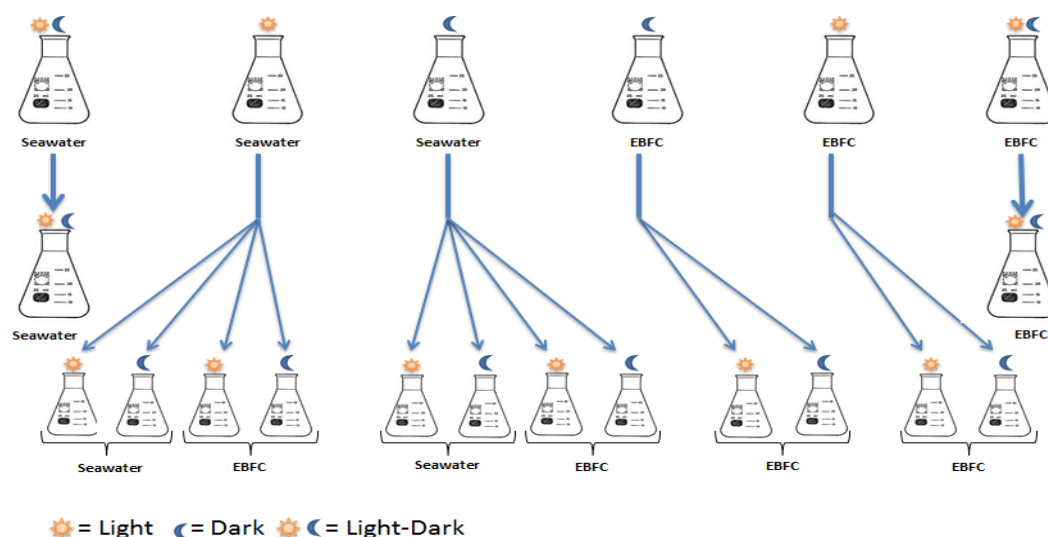


Fig. 3.4.1: *P. salmonis* 5692, 5892 or 5896 was cultured in 10 ml EBFC or seawater. After reached an appropriate OD₆₀₀, the bacteria were diluted in EBFC or seawater, under different access to light. OD₆₀₀ was measured three times a day, and checked regularly for contaminations. (Figure made by Julia Tandberg).

3.5 Colony Forming Units

P. salmonis 5692, 5892 or 5896 was cultured in 10 ml of liquid media and incubated at 20 °C with gentle shaking (100 rpm). After growth to optimal inoculum, the cultures were diluted in 50 ml media and OD₆₀₀ was measured three times a day. Serial dilutions were made of each OD₆₀₀ and 10 µl of each dilution were plated onto the same ECA plate. The plates were incubated at 20 °C and the colony forming units (CFU) were counted after approximately 1-2 weeks.

3.6 Plasmid isolation

P. salmonis 5692, 5892 or 5896 was grown in 10 ml media at 20 °C and 100 rpm to a dense culture in the middle of exponential phase. The bacteria were centrifuged at 4500 rpm for 5 minutes in a Rotina 420R (Hettich zentrifugen, Buckinghamshire, UK) and the presence of natural occurring plasmids were evaluated by following the manufacturer's instructions in the E.Z.N.A® Plasmid Mini Kit I (Omega Bio-Tek) (see appendix B: Kit protocols). Plasmid concentration and purity were measured with the Picodrop spectrophotometer and visualized on a 1% agarose gel.

3.7 Isolation and analysis of outer membrane vesicles

Outer membrane vesicles (OMVs) were isolated from 100 ml dense cultures in the late exponential growth phase of *P. salmonis* 5692, 5892 or 5896. Cells were removed from suspension by centrifugation at 11500 rpm in an AvantiTM J-25 cooling centrifuge (Beckman Coulter Inc., CA, USA) at 4 °C for 10 minutes. The supernatant was collected and filtered through a 0.45 µm filter (GE Healthcare Life Sciences, Buckinghamshire, UK) and a 0.22 µm filter (GE Healthcare). The supernatant was then ultra-centrifuged in a Sorvall®DiscoveryTM 100 (Sorvall products, L.P, Newtown, USA), 37 ml supernatant per tube, for 2 hours at 25900 rpm, 4 °C. The supernatant was discarded, and the pellet was washed with 37 ml cold 50 mM N-2-hydroxyethylpiperazine-N9-ethanesulfonic acid (HEPES) buffer pH 6.8 (see appendix A:

Solutions). The samples were ultra-centrifuged for an additional 30 minutes at 25900 rpm (4 °C) to pellet the OMVs. The supernatant was discarded and the OMVs were resuspended in 100 µl 1x phosphate buffered saline (PBS) pH 7.2 (see appendix A: Solutions). OMV aliquots (25 µl) were then stored at -80 °C for later use. In addition, one aliquot was spread on an ECA plate and incubated at 20 °C for at least three weeks to ensure sterility.

To check the content of the OMVs, aliquots were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The OMVs were mixed with sampling buffer (see appendix A: Solutions), and 20 µl of this mix was loaded onto a gel (Thermo Scientific). The gel was run at 60V for 15 minutes, followed by 100V for 1 hour. The gel was then moved to a container for Coomassie-Blue staining (see appendix A: Solutions) and destaining (see appendix A: Solutions). Subsequently, the gel was rinsed with MQ water and viewed in a Molecular Imager[®] Gel Doc[™] XR+ (Bio-Rad Laboratories, Inc., CA, USA). In addition, the protein contents of the OMVs (non-dilution, 10x and 100x dilutions of samples) were electro blotted onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare). The membrane was then blocked in 5 % skim milk (BD Difco) for 1 hour at room temperature on a Stuart roller mixer SRT9 (Bibby Scientific, Staffordshire, UK). Primary antibodies (Rabbit-anti-*P. salmonis* K231) were a kind gift from Duncan J. Colquhoun and was used to immunoblott the membrane at 4 °C overnight and thereafter incubated in Goat Anti-rabbit IgG (H+L)-HRP conjugate (Bio-Rad) for 1 hour at room temperature. Luminata[™]Crescendo Western HRP Substrate detection solution (EMD Millipore, MA, USA) was added onto the membrane to give enhanced chemiluminescence and incubated for 5 minutes. Excess of fluid was removed and the membrane was wrapped in plastic to prevent it from drying out. The membrane was then exposed and viewed in a Chemi Genius 2 Bio Imaging System (Syngene, Cambridge, UK).

3.8 Electron microscopy of *P. salmonis* and outer membrane vesicles

P. salmonis cell surface structures were analyzed by using transmission electron microscopy (TEM) and scanning electron microscopy (SEM) in collaboration with the Electron microscopy unit at the Department of Bioscience, UiO.

Analysis of cell surface structures in TEM; *P. salmonis* 5692, 5892 or 5896 from liquid culture, directly from plate and fixation in liquid culture with a mix of 4 % paraformaldehyde (PFA) (Sigma-Aldrich) and 0.5 % glutaraldehyde (Sigma-Aldrich) were tested. The bacteria were centrifuged and resuspended in 0.2M cacodylate buffer (see appendix A: Solutions). The bacteria suspensions were then incubated with Carbon coated Formvar copper grids (200 mesh, hexagonal) (Electron microscopy unit, IBV, UiO) for 10 minutes. The grids were washed three times with cacodylate buffer, four times with distilled water (dH₂O), and negatively stained with 1.5 % uranyl acetate (UA) for 2 seconds. Then the grids were washed once with dH₂O and finally stabilized with (9:1) methyl-cellulose in UA for 10 minutes on ice. The grids were then dried and viewed in a Phillips CM200 transmission electron microscopy.

Analysis of cell surface structures in SEM; *P. salmonis* 5692, 5892 or 5896 from liquid culture and directly from plate were incubated on pre-coated poly-lysine (Sigma-Aldrich) coverslips (Thermo Scientific). The coverslips were then fixed overnight at 4 °C with 2 % glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.4. They were then washed two times in the same buffer for 10 minutes each. The samples were dehydrated in a graded ethanol series for 10 minutes at 70, 90, 96 and 100 % and 15 minutes 4 rinsing in 100 % ethanol. Dehydrated samples were subsequently critical-point dried using carbon dioxide in a CPD 030 critical-point dryer (Bal-Tec, CA, USA), then mounted on stub with carbon-circles colloidal silver and sputter coated with a Cressington coating system 308R. The samples were then viewed in a Hitachi S-4800 scanning electron microscopy, and the images were acquired using Scandium software.

The isolated OMVs were viewed in TEM; Carbon coated Formvar copper grids (100 mesh, hexagonal) (Electron microscopy unit, IBV, UiO) were incubated on an OMV solution from *P. salmonis* 5692, 5892 or 5896 for 5 minutes and then washed three times with PBS. Accordingly, the samples were fixed in 1 % glutaraldehyde (Sigma-Aldrich) for 4 minutes, washed three times with PBS, two times with Milli-Q (MQ) water, negatively stained for 1 minute with 4 % UA (Sigma-Aldrich), washed once with MQ water and finally stabilized with a solution of (9:1) methyl-cellulose (Sigma-Aldrich) in UA for 10 minutes on ice. The grids were then dried and viewed in a Philips CM200 transmission electron microscope and the images were acquired using iTEM software. Professor Norbert Roos from the Electron

microscopy unit at the Department of Bioscience (UiO) handled the microscope and took the images of the bacteria and the isolated OMVs.

3.9 Infection of CHSE-214 cells

Chinook salmon embryo-214 cell line (CHSE-214) were seeded onto coverslips (Thermo Scientific) in six-well plates (Thermo Scientific) with cell growth medium (3 ml per dish): Leibovitz L-15 medium (Bio Whittaker, Wokingham, UK) supplemented with 10 % fetal bovine serum (FBS) (Bio Whittaker), 40 μ M β -Me (Gibco BRL, Uxbridge, UK), 4 mM L-glutamine (Gibco) and 50 μ g/ μ l gentamycin sulfate (Bio Whittaker). Each well had a density of 100 000 cells and they were incubated at 20 °C in a Termaks incubator (Thermo Scientific) for 6 days to reach a 50-70 % confluence. Prior to infection with *P. salmonis* 5692, 5892 or 5896 the cell growth media were removed and replaced with media not containing gentamycin sulfate. Bacteria from EBFC liquid cultures were centrifuged at 7176 g for 2 minutes, resuspended in cell growth medium without gentamycin sulfate, and adjusted to OD 0.6 before 200 μ l bacterial suspension was added to each well. The cells were then incubated in medium without gentamycin sulfate at 20 °C in the Innova 4230 incubator under different light and dark conditions until 2, 5 or 10 days post-infection. The cells were fixed using 4 % PFA (Sigma-Aldrich) and then kept in PBS. The cells were permeabilized for 5 minutes with 0.1 % triton X-100 (Sigma-Aldrich) in PBS, followed by two times washing with 0.01 % TWEEN® 20 detergent (Calbiochem, Merck, Germany) in PBS. The cells were then blocked for 30 minutes with 5 % goat serum (Molecular probes by life technologies, Invitrogen) in 0.01 % TWEEN® 20 (blocking solution). Rabbit-anti-*P. salmonis* was diluted in blocking solution (1:200) and cells were incubated in the solution for 1 hour at room temperature. Subsequently, the cells were stained with a solution of goat-anti-rabbit Alexa green 488 antibody (1:500) (Molecular probes®, Invitrogen), wheat germ agglutinin (WGA) (1:250) (Molecular probes®, Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI) (1:1000) (Molecular probes®, Invitrogen) in blocking solution for 1 hour at room temperature. Cells were washed twice with 0.01 % TWEEN® 20 and coverslips were mounted on objective glasses (Thermo Scientific) with 1 drop ProLong® gold antifade reagent (Molecular Probes®, Invitrogen). The objective glasses were incubated at room temperature in the dark overnight, and for long term storage at 4 °C in the dark. The cells were then analyzed further in a

fluorescence microscope and confocal microscope (see 3.14 Fluorescence microscopy section).

3.10 Zebrafish embryo care and maintenance

Zebrafish (*Danio rerio*) embryos used in this study were purchased from Peter Aleström's zebrafish facility at The Department of Basic Sciences and Aquatic Medicine, Norwegian University of Life Sciences. Different zebrafish embryos strains were used; the wild type strain AB, casper (*roy*^{-/-}; *nacre*^{-/-}) strain and other transgenic zebrafish strains expressing red fluorescence in neutrophils Tg(LysC:DsRED2) and macrophages Tg(mpeg1:gal4ff): (UAS:nfsB-mCherry). Zebrafish eggs were collected in a tube with 28 °C embryo water (see appendix A: Solutions) and wrapped in heat packs for transportation. The eggs were then transferred from Peter Aleström's zebrafish facility and kept at 28 °C in the Griffiths Zebrafish Lab at the University of Oslo. In addition, the wild type strain AB embryos used in this study were produced in-house. Zebrafish embryos were manually dechorionated at the age of 30 hours post fertilization, and kept at 28 °C prior to injections.

3.11 Infection of zebrafish embryos with *P. salmonis*

Overnight cultures of *P. salmonis* 5692, 5892 and 5896 were harvested by centrifugation at 10 000 g for 10 minutes at 4 °C (Mikro200R, Hettich zentrifugen), resuspended in PBS pH 7.4 and optical density at 600 nm (OD₆₀₀) was measured by a Bio Photometer (Eppendorf AG). The OD₆₀₀ was adjusted to a desired value with PBS and then phenol red sodium salt solution (Sigma-Aldrich Co) was added to a final concentration of 0.01 % to help visualize the injections.

Injection needles (without filament, outer and inner diameter of 1.0 and 0.78 mm, length of 100 mm, Harvard Apparatus, Holliston, MA, USA) were made using a Sutter needle puller (P-97 Flaming/Brown micropipette puller, Novato, CA, USA). The needle puller was installed on the following settings: delay-110, velocity-50, pull-40, heat-610 and pressure-500.

The needle was loaded with 10 µl bacterial suspension containing phenol red by using a needle-loading pipette (Eppendorf). Then the needle was attached to a micromanipulator (Narishige, Tokyo, Japan) and a pressure controller ((FemtoJet, Eppendorf). To unseal the

needle, the needle was submerged in mineral oil (Sigma) and the tip was broken off by using sharp forceps. The volume was determined by doing trial injections into the oil, and the diameter of the red droplets was measured with a ruler incorporated into an eyepiece of the microscope. The volume for infection of the embryos should be approximately 1 nl. The wild type strain AB or the transgenic zebrafish embryos 48 hours post fertilization (hpf) were anesthetized using a water bath supplemented with approximately 170 $\mu\text{g/ml}$ Tricaine methanesulfonate MS-222 (Tricaine) pH 7.4 (Argent Laboratories Group, Inc., Redmond, WA, USA). After 1-2 minutes, the embryos were transferred to a 2 % agarose (BD) injection plate and excess water was removed to immobilize the fish. Fish were injected in the Duct of Cuvier (Fig. 3.11.1), and the injections were deemed successful if the phenol red could be observed in the blood circulation following injection (Fig. 3.11.2).

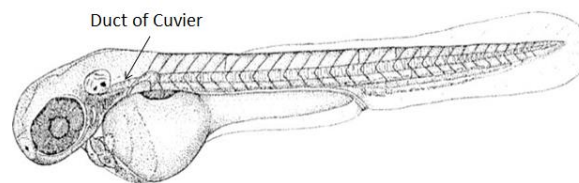


Fig. 3.11.1: Zebrafish embryo 48 hpf with an arrow showing the injection site in this study; Duct of Cuvier. Figure is taken from: <http://www.neuro.uoregon.edu/k12/Part%202.html> .

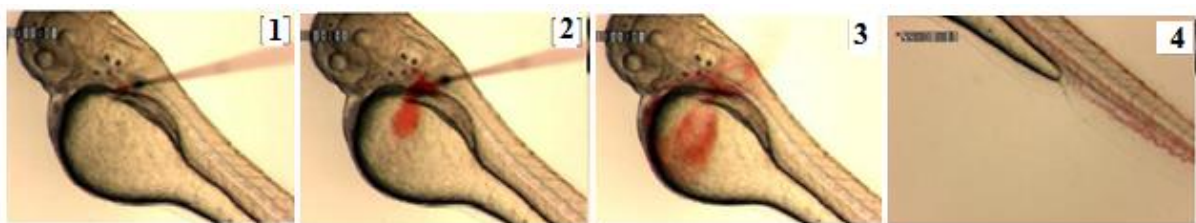


Fig. 3.11.2: Successful infection of a zebrafish embryo; can see the phenol red spreading into the blood circulation. This figure is made by David Westmoreland during his Master's thesis in Gareth Griffiths group (UiO).

Infected fish were transferred into new petri dishes with fresh embryo water and kept at 22 °C. The embryos in this study were used for monitoring survival, visualization of bacterial infection route, whole-mount immunohistochemistry or gene expression analysis by quantitative PCR. For survival experiments, approximately 20 embryos per group were injected with *P. salmonis* 5692, 5892 or 5896 in PBS with OD₆₀₀ of 0.5 and 2.0. In addition PBS without bacteria supplemented with phenol red was used for injections as negative controls.

3.12 Calcein AM staining of *P. salmonis* 5692 and injections into zebrafish embryos

P. salmonis 5692 was centrifuged at 7176 g for 2 minutes, resuspended in 1 ml PBS pH 7.4 and OD₆₀₀ was adjusted to OD₆₀₀ 2.0 in 500 µl PBS. The suspension was then incubated for 15-20 minutes with 30 µl acetoxymethyl diacetylester of calcein (Calcein AM) (1 mM Calcein AM (Molecular Probes®, Invitrogen) in dimethyl sulfoxide (DMSO) (Sigma-Aldrich®). Approximately 10 zebrafish embryos of each strain Tg(LysC:DsRED2) and Tg(mpeg1:gal 4ff):(UAS:nfsB-mCherry) was injected in the Duct of Cuvier with the bacterial suspension supplemented with phenol red by Lilia Ulanova. The embryos were then analyzed further in a confocal microscope after 1 hour (see 3.14 Fluorescent microscopy section).

3.13 Whole-mount immunohistochemistry of zebrafish embryos

Wild type strain AB zebrafish or casper (*roy*^{-/-}; *nacre*^{-/-}) embryos were injected in the Duct of Cuvier with *P. salmonis* 5692, 5892, 5896 or with PBS as controls. The embryos were incubated at 22 °C, and the wild type strain AB zebrafish were in addition reared in 0.003% of 1-phenyl-2-thiourea (PTU) (Sigma), to reduce the pigmentation. At day 0, 1, 3 and 6 post infections, the embryos were euthanized in ice bath, and in tricaine bath. The embryos were then washed in PBS and fixed in 2 % trichloroacetic acid (TCA) (TCA powder in dH₂O) for 3 hours at room temperature. After fixation, the embryos were washed carefully three times for 5 minutes with PBS, and stored for long term storage at 4 °C in 500 µl PBS.

In this study, 5 fish were used for each days post infection (dpi) and each bacterial strain. The embryos were transferred into an 8-well lab-Tek® glass chamber slide (Thermo Scientific) with 2-3 fish per each chamber, washed twice with PBT (0.8 % triton X-100 in PBS) at room temperature and chilled on ice. PBT was then replaced with 200-300 µl cold 0.25 % trypsin (2.5 % Trypsin (10X), GIBCO®, Invitrogen) in PBT, and incubated on ice for 4 minutes (2-3 days old embryos), 5 minutes (3-4 days old embryos) or 6 minutes (5-6+ days old embryos). The trypsin solution was immediately replaced with PBT supplemented with 10 % goat serum (blocking solution) and incubated at room temperature for 5 minutes. The embryos were then washed three times for 5 minutes at room temperature. Nonspecific binding sites were blocked with 10 % goat serum in PBT for 1 hour at room temperature on a GFL 3005 orbital-

shaker (DJB Labcare, Buckinghamshire, UK) with gentle agitation around 40 rpm. In the meantime, the primary antibody (Rabbit-anti-*P. salmonis*) was diluted in PBT with 10 % goat serum (1:200). Subsequently, the blocking solution was replaced with diluted primary antibody, and incubated overnight at 4 °C on a rocking table with gentle agitation. The antibody was then removed, and washed over several hours with PBT. PBT was changed at least five times, and finally the embryos were washed with PBS. The secondary antibody (Goat-anti-rabbit Alexa red 594) was then diluted in PBT containing 10 % goat serum (1:500), and the PBS were replaced with the secondary antibody solution and the samples were incubated overnight at 4 °C with gentle agitation. The embryos were kept in the dark from this stage, to avoid bleaching of the samples. Subsequently, the antibody was removed and washed with PBT over several hours with five changes of PBT. Finally, PBT was removed and embryos were stained with DAPI in PBS (1:1000), and incubated at room temperature for 10 minutes. The DAPI was then removed, and PBS added to the embryos. The zebrafish embryos were then analyzed further in a fluorescence and confocal microscope (see 3.14 Fluorescent microscopy section).

3.14 Fluorescence microscopy

Wholemount samples and some of the CHSE-214 cells were analyzed in a Nikon Eclipse TE300 fluorescence microscope (Nikon Instruments, NY, USA). The samples were viewed by using a Nikon Intensilight C-HGFI (Nikon Instruments), and images were acquired using NIS-Elements BR 3.0 software (Nikon Instruments). The images were then further analyzed on the Fiji platform.

Olympus FluoviewTM FV1000 Confocal Microscope (Olympus Europa Holding GmbH, Hamburg, Germany) was also used to do live-imaging of zebrafish embryos infected with calcein AM stained *P. salmonis* 5692 and to analyze the CHSE-214 cells. Lilia Ulanova at the Department of Bioscience (UiO) handled the microscope and took the images. Images were acquired using Olympus FluoviewTM version 3.1 software (Olympus Europa Holding GmbH) and further processed on the Fiji platform. Fluorophores used to study the cell infection was 488 (Alexa green), 594 (WGA) and 405 (DAPI). On the day of analysis five random overview pictures were taken from each strain of *P. salmonis* at day 5 post infection, in light and dark respectively and controls. The number of bacteria per cell was counted manually, to investigate a possible difference in infection rate in light versus dark. In addition, two pictures

from each sample were taken at a higher magnification (60X) to confirm that the bacteria were inside the cells. In addition, wholemount samples were further analyzed by three dimensional confocal imaging at NVI in corporation with Even Thoen. A Zeiss LSM 710 confocal microscope (Carl Zeiss, Jena, Germany) was used to assess and visualize the bacteria and staining; images were acquired using LSM software ZEN (Carl Zeiss) version 2009. Fluorescence from DAPI was excited at 405 nm by a Diode laser and fluorescence from Alexa 594 at 514 nm by an Agron laser.

3.15 Zebrafish embryo immune response to *P. salmonis* 5692 infection

Zebrafish embryos Tg(LysC:DsRED2) were injected with calcein AM-stained *P. salmonis* 5692 and Tg(mpeg1:gal4ff):(UAS:nfsB-mCherry) embryos were injected with calcein AM-stained and non-stained *P. salmonis* 5692. In addition, PBS was injected as a control. At each time point (day 1 and 6 post infection, plus PBS control), 9 randomly chosen zebrafish embryos from each group was euthanized by an overdose of Tricaine solution (200-300 µg/ml). The embryos were transferred into 1.5 ml Eppendorf tubes (Eppendorf), three embryos in each tube. Then, the embryo water was replaced by RNALater (Ambion by Life Technologies™, Carlsbad, CA, USA) immediately after the transfer. The samples were kept for long term storage at 4 °C until extraction of RNA. For the extraction of total RNA, RNALater was replaced with 600 µl buffer RLT (supplemented in RNeasy Mini Kit, QIAGEN). The samples were homogenized by using a mortar and pestle (Sigma-Aldrich), followed by a blunt 20 gauge needle fitted to a small 1 ml syringe (BD), and the lysate was passed at least five times through the needle on the syringe. Subsequently, the samples were centrifuged at full speed for 3 minutes (Mikro200R) and the RNA-containing supernatant was mixed with an equal volume of 70 % ethanol (Kemetyl, Vestby, Norway), and loaded on RNeasy Mini Spin columns (QIAGEN GmbH, Hilden, Germany). The samples were thereafter handled according to the manufacturer's instruction, including a 15 minute on-column DNase treatment using RNase-free DNase set (QIAGEN) (see appendix B: Kit protocols). RNA was finally diluted in 30 µl RNase-free H₂O (QIAGEN). RNA quantity and quality was measured with a Picodrop spectrophotometer. Reverse transcription reaction was performed by using High Capacity RNA to cDNA kit (Applied Biosystems) (see appendix B: Kit protocols). Maximum amount of 9 µl RNA per reaction was used. In addition, negative

control of each sample was made to confirm the absence of genomic DNA. The samples were handled according to the manufacturer's instruction, including incubation of the reaction at 37 °C for 1 hour, stopping the reaction by heating to 95 °C for 5 minutes and then hold at 4 °C. This was done in a GeneAmp PCR system 2700 PCR machine and cDNA were ready for quantitative PCR (qPCR) or could be kept for long-term storage at -20 °C.

Primers (Table 3.15.1) were chosen to target a repertoire of the immune response from zebrafish: TNF- α , IL-1 β , IL-8, IL-10, IFN γ 1-2 and socs3b, in addition the reference genes 18S rRNA and EF1 α were used to normalize the relative transcription level.

Table 3.15.1: Primers used for qPCR in the experiment.

Gene name	Gene symbol	Manufacturer
<i>18S rRNA</i>	<i>zgc:158463</i>	Invitrogen
<i>elongation factor 1-alpha</i>	<i>ef1a</i>	Invitrogen
<i>tumor necrosis factor a</i>	<i>tnfa</i>	QIAGEN
<i>interleukin 1, beta</i>	<i>il1b</i>	QIAGEN
<i>interleukin 8</i>	<i>il8</i>	QIAGEN
<i>interleukin 10</i>	<i>il10</i>	QIAGEN
<i>interferon, gamma 1-2</i>	<i>ifng1-2</i>	QIAGEN
<i>suppressor of cytokine signaling 3b</i>	<i>socs3b</i>	QIAGEN

The primers were used in a concentration of 5 μ M, and the cDNA was diluted (1:10) in MQ-water. Quantitative PCR was performed in duplicates using a Lightcycler® 480 (Roche, Basel, Switzerland) qPCR machine (software: Lightcycler® 480 version 15.1.62) in 20 μ l reactions volume containing Lightcycler® 480 SYBR Green I Master (Roche), 0.5 μ M of each primer and 5 μ l template. The qPCR machine was set at following conditions: 5 minutes denaturation at 95 °C, 45 cycles amplification with 10 seconds at 95 °C, 30 seconds at 60 °C and 8 seconds at 72 °C. The cycle threshold (CT) values were analyzed in Microsoft Excel 2010, in addition to analysis of melting curve to verify single amplification peaks. Statistical analyses were performed in GraphPad Prism 6.

4 Results

4.1 Growth studies and identification of *P. salmonis* by 16S rRNA sequencing

The different *P. salmonis* strains were obtained from the Veterinary Institute (NVI) (Oslo). 16S rRNA sequencing was then performed on *P. salmonis* to ensure that further experiments include the correct bacterium. After the 16S rRNA sequencing, the sequences were obtained from ABI-lab, IBV, UiO and analyzed by the basic local alignment tool (BLAST). According to the data (Fig. 4.1.1), the strains used in this study was identified as *P. salmonis* with a 98 % identity, when the query sequence was compared to sequences in the database. All three *P. salmonis* strains were analyzed, but only the data for *P. salmonis* 5692 (NVI 5692) is presented here as they all gave similar result. Sequences for the other two *P. salmonis* strains are presented in appendix (see appendix C: Other results).

Description	Max score	Total score	Query cover	E value	Ident	Accession
Piscirickettsia salmonis strain CGA/ID556/2012 16S ribosomal RNA gene, partial sequence	241	241	100%	2e-60	98%	KC473451.1
Piscirickettsia salmonis strain CGA/ID595/2012 16S ribosomal RNA gene, partial sequence	241	241	100%	2e-60	98%	KC473449.1
Piscirickettsia salmonis strain CGA/8417/2012 16S ribosomal RNA gene, partial sequence	241	241	100%	2e-60	98%	KC473442.1
Piscirickettsia salmonis strain CGA/1850/2012 16S ribosomal RNA gene, partial sequence	241	241	100%	2e-60	98%	KC473436.1
Piscirickettsia salmonis strain CGA/1881/2012 16S ribosomal RNA gene, partial sequence	241	241	100%	2e-60	98%	KC473433.1
Piscirickettsia salmonis strain CGA/8998/2012 16S ribosomal RNA gene, partial sequence	241	241	100%	2e-60	98%	KC473432.1
Piscirickettsia salmonis strain CGA/ID495/2012 16S ribosomal RNA gene, partial sequence	241	241	100%	2e-60	98%	KC473430.1
Piscirickettsia salmonis strain CGA/ID432/2012 16S ribosomal RNA gene, partial sequence	241	241	100%	2e-60	98%	KC473426.1
Piscirickettsia salmonis strain CGA/8076/2012 16S ribosomal RNA gene, partial sequence	241	241	100%	2e-60	98%	KC473425.1
Piscirickettsia salmonis strain NVI 5910 16S ribosomal RNA gene, partial sequence	241	241	100%	2e-60	98%	EU170262.1
Piscirickettsia salmonis strain NVI 5895 16S ribosomal RNA gene, partial sequence	241	241	100%	2e-60	98%	EU170261.1
Piscirickettsia salmonis strain NVI 5894 16S ribosomal RNA gene, partial sequence	241	241	100%	2e-60	98%	EU170260.1
Piscirickettsia salmonis strain NVI 5892 16S ribosomal RNA gene, partial sequence	241	241	100%	2e-60	98%	EU170259.1
Piscirickettsia salmonis strain NVI 5891 16S ribosomal RNA gene, partial sequence	241	241	100%	2e-60	98%	EU170258.1
Piscirickettsia salmonis strain NVI 5692 16S ribosomal RNA gene, partial sequence	241	241	100%	2e-60	98%	EU170254.1

Fig. 4.1.1: Sequences alignments for *P. salmonis* 5692 from BLAST.

Alignments of the query sequence (138 bp) with the subjected sequence for *P. salmonis* 5692 (NVI 5692) (420 bp) is shown in more details in Fig. 4.1.2. There are only three nucleotides in the query sequence that do not fit to the subjected sequence in the database. Therefore, *P. salmonis* 5692 used in this study, are *P. salmonis* 5692 with a 98 % identity. However, the

subjected sequence in the database is a partial sequence and there may be uncertainties around the sequence. In addition, the 138 bp query sequence is only matching a part of the 420 bp subjected sequence.

Score	Expect	Identities	Gaps	Strand
241 bits(266)	2e-60	135/138(98%)	0/138(0%)	Plus/Minus
Query 1	GGGTTCCCCCATYGTCCAATATTCCCCACYGCTGCCTCCCGTAGGAGTCTGGGCCGTGT	60		
Sbjct 335	GGGTTCCCCCATTTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGT	276		
Query 61	CTCAGTCCCAGTGTGGCTGGCCATTCTCTCAAACAGCTATGGATCGTCSCTTGGTAAG	120		
Sbjct 275	CTCAGTCCCAGTGTGGCTGGCCATTCTCTCAAACAGCTATGGATCGTCSCTTGGTAAG	216		
Query 121	CCTTTACCCTACCAACTA	138		
Sbjct 215	CCTTTACCCTACCAACTA	198		

Fig 4.1.2: Sequence obtained after 16S rRNA sequencing from *P. salmonis* 5692 (query sequence) is compared to a subjected sequence in the database. The subjected sequence already in the database was isolated in another study (Mikalsen et al., 2008) from *P. salmonis* strain NVI 5692 16S ribosomal RNA gene, partial sequence. Y and S in the sequence alignment means that more than one kind of a nucleotide can occur at that position: Y = nucleotide T or C and S= nucleotide G or C.

After the confirmation of *P. salmonis* by 16S rRNA sequencing, growth studies were performed. Due to the scarce information available as to how to grow the bacterium, different media such as CHAB– and ECA plates in addition to Eugon broth with and without iron and casamino acids at different temperatures were tested. Optimal growth was obtained by using the EBFC liquid medium and ECA plates and the ideal temperature of *P. salmonis* was determined by measuring growth at 18 °C, 20 °C and 23 °C. Of the incubation temperature tested, no growth was observed for any of the three *P. salmonis* strain at 23 °C while growth were visible at 18 °C after 5 days incubation on ECA plates. Optimal growth was observed at 20 °C, and individual colonies were then visible after 5-7 days incubation. The colonies were around 1 mm in diameter, slightly convex, shiny and grey-white. In the EBFC liquid medium the cultures went from bright, clear, yellow color to a dense and opaque color at 20 °C after 2-4 days incubation, with a shaking of 100 rpm.

4.2 Antibiotic susceptibility

Establishment of how *P. salmonis* respond to different antibiotics *in vitro* was tested with the disc-diffusion method on ECA plates incubated at 20 °C (Table 4.2.1). A zone of sensitivity was measured around each tablet; the larger the area of no growth surrounding the tablet the

more sensitive the bacteria were to the drug. The data suggested that all three strains of *P. salmonis* were sensitive to almost all of the antibiotics tested, except for sulfadiazine. *P. salmonis* 5896 was in addition resistant to sulfamethoxazole/trimethoprim and sulfamethoxazole.

Table 4.2.1: Antibiotic susceptibility test: Various antibiotics were tested for *P. salmonis* 5692, 5892 and 5896. The star symbol (☆) = the diameter of the no growth zone around the antibiotic tablet was too large to measure due to *P. salmonis* sensitivity towards the various antibiotics.

Antibiotic	Tablets of	<i>P. salmonis</i> 5692		<i>P. salmonis</i> 5892		<i>P. salmonis</i> 5896	
		Zone of no growth	Resistance	Zone of no growth	Resistance	Zone of no growth	Resistance
Sulfisoxazole	250 µg	☆	-	☆	-	☆	-
Chloramphenicol	30 µg	☆	-	☆	-	☆	-
Mecillinam	10 µg	☆	-	☆	-	☆	-
Gentamycin	30 µg	☆	-	☆	-	☆	-
Sulfamethoxazole/trimethoprim	23.75µg/1.25 µg	☆	-	☆	-	Growth	+
Oxacillin	5 µg	☆	-	☆	-	☆	-
Tetracyclin	30 µg	☆	-	☆	-	☆	-
Ciprofloxacin	5 µg	☆	-	☆	-	☆	-
Ampicillin	10 µg	☆	-	☆	-	☆	-
Sulfadiazine	250 µg	Growth	+	Growth	+	Growth	+
Cephalexin	30 µg	3.0 cm	-	3.5 cm	-	3.0 cm	-
Sulfamethoxazole	23.75 µg	☆	-	☆	-	Growth	+
Rifampicin	5 µg	1.5 cm	-	1.0 cm	-	0.8 cm	-

4.3 Growth curves and CFU

It has been shown that optimal growth is dependent on the size of the inoculum (Augustin et al., 2000). An inoculum is the number of bacteria used in an inoculation of a new liquid culture. To test whether the inoculum had an effect on the growth of *P. salmonis*; different

OD₆₀₀ was used to inoculate a new liquid culture and growth was measured. The results from the inoculum test for the three *P. salmonis* strains are shown in Fig. 4.3.1.

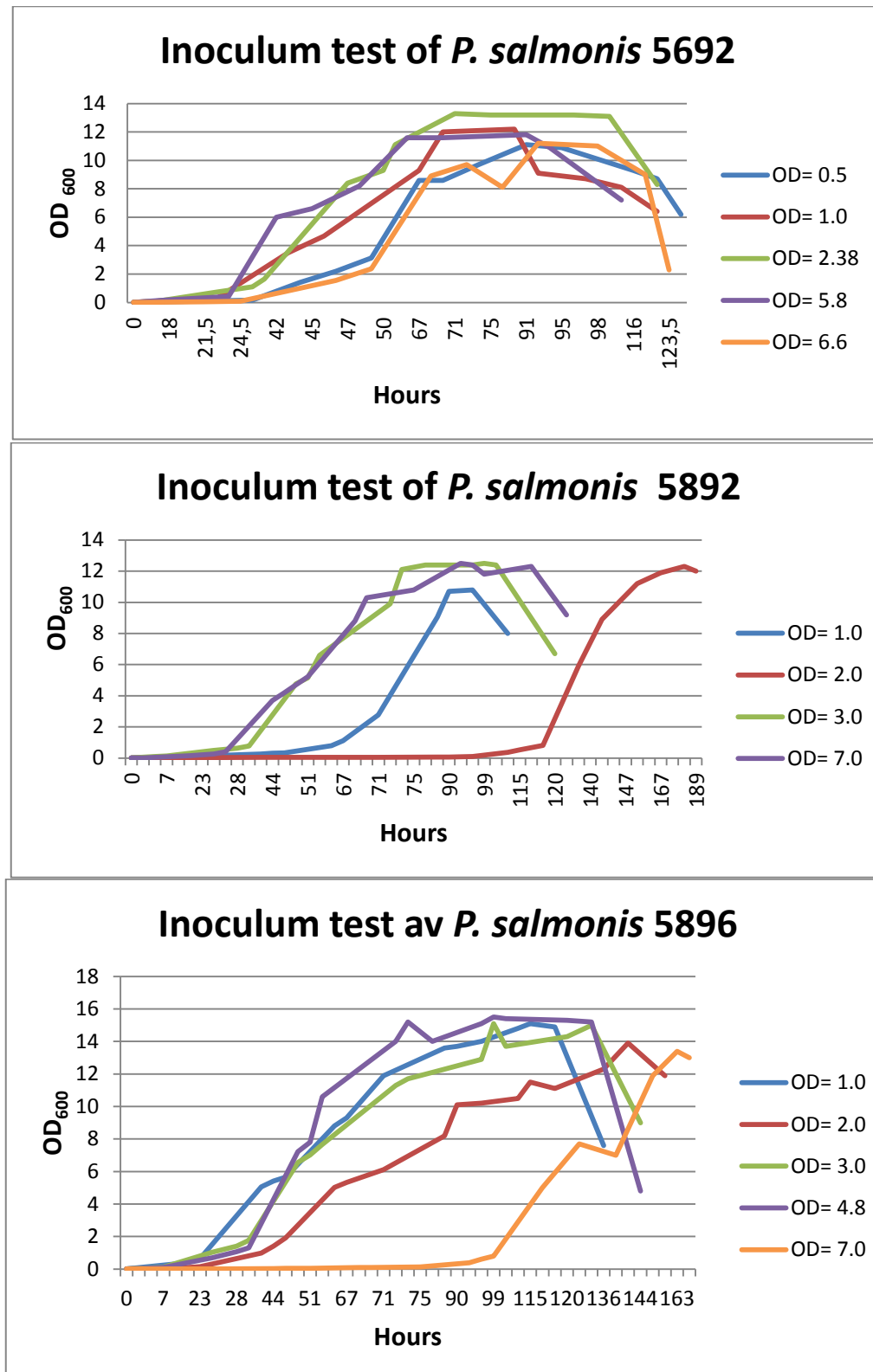


Fig. 4.3.1: Inoculum test of *P. salmonis* strains to find optimal OD₆₀₀ to use as an inoculum for growth curves.

P. salmonis 5692 and 5896 needed an inoculum of OD₆₀₀ 2.38 and 4.8 respectively, and *P. salmonis* 5892 needed a higher inoculum between OD₆₀₀ 3 and 7. Based on the optimal OD₆₀₀ of the inoculum, growth curves were made of all three *P. salmonis* strains (Fig. 4.3.2). According to Fig. 4.3.2 *P. salmonis* 5896 has a lag phase from 0-25 hours, exponential phase from 26-47 hours, stationary phase from 48-72 hours and then it reaches the death phase. The stationary phase begins at OD₆₀₀ 12 and ends around OD₆₀₀ 15. *P. salmonis* 5892 is slower growing than the other two strains, as it needs more time to reach the high OD₆₀₀ values.

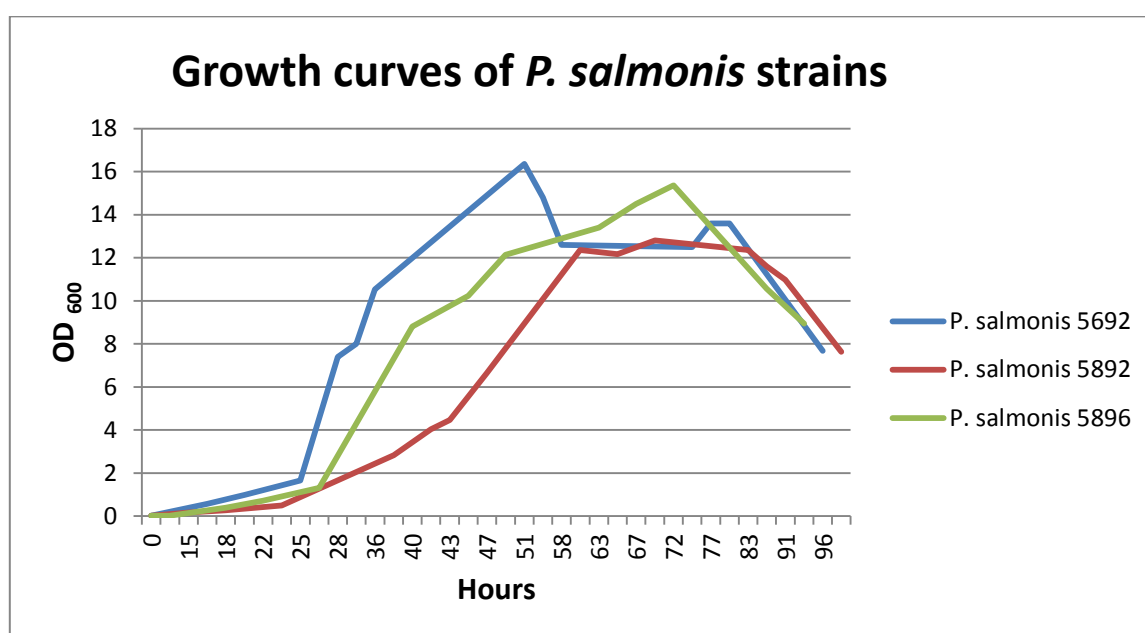


Fig. 4.3.2: Comparison of growth curves between *P. salmonis* 5692, 5892 and 5896. All cultures were maintained in EBFC medium in light at 20° C and 100 rpm (n= 3).

When growth measurements were performed, an interesting observation was noticed. It seemed like *P. salmonis* grew better in the dark and slower in the light. An experiment was therefore performed to further evaluate the observation. The study was based on Gómez-Consarnau's work where they demonstrated that a *Vibrio* strain was dependent on light for survival in stress conditions such as seawater (Gómez-Consarnau et al., 2010). The experiment set up is showed in Fig. 3.4.1, and the results after starvation in seawater (SW) and during alterations in access to light are shown in Fig. 4.3.3. According to Fig. 3.4.1, some of the bacteria from each strain were only cultured in seawater. No growth was observed or measured for these bacteria (Fig. not shown). The same was the case for *P. salmonis* 5896, which were first cultured in seawater, then in EBFC. This experiment was only tested once, compared to other experiments which were at least performed three times. However, according to Fig. 4.3.3, *P. salmonis* could survive in seawater for a period of time, and during

this period they preferred the light. However, after transferring the bacteria from the starvation period in seawater to EBFC medium, they preferred the dark.

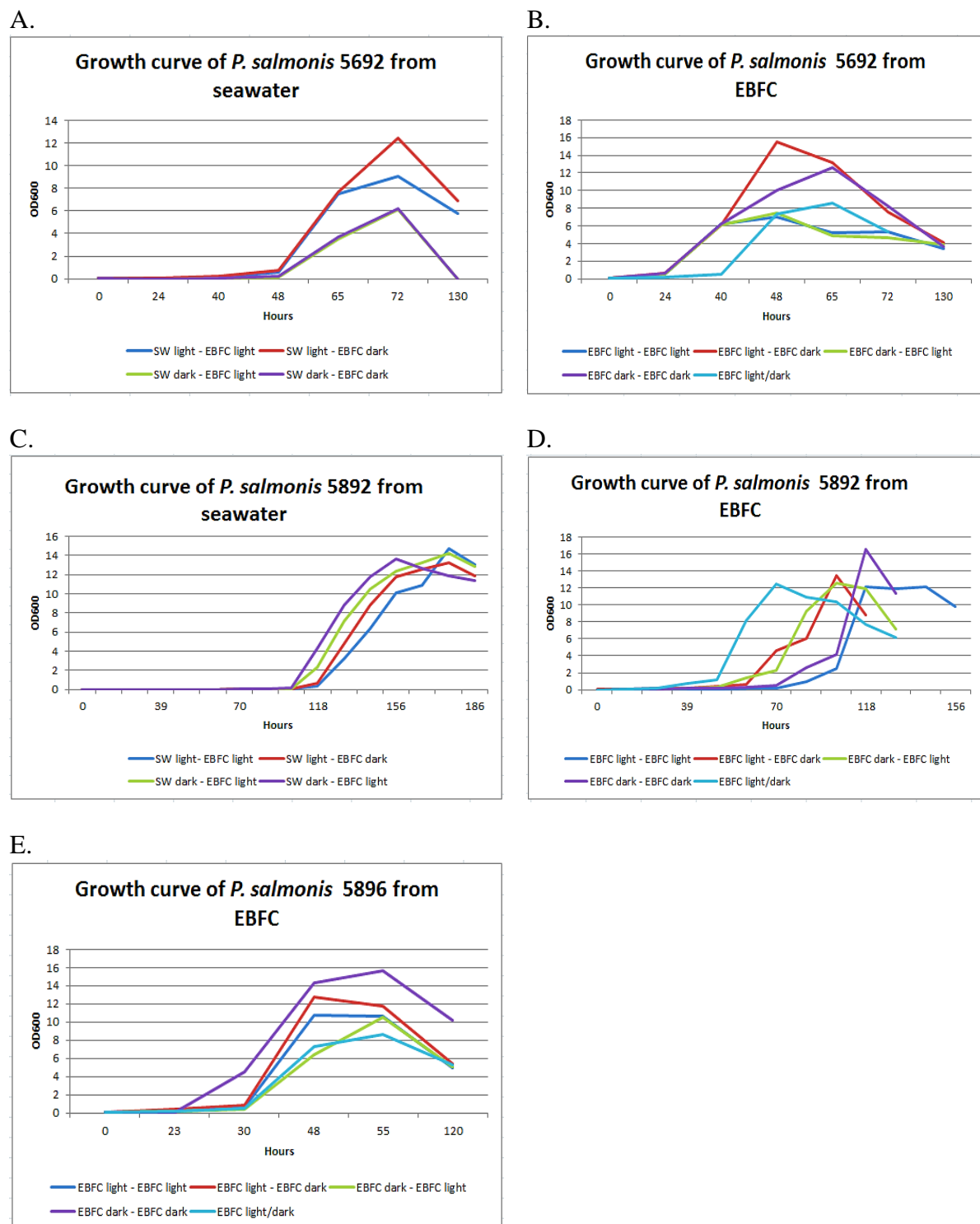


Fig. 4.3.3: Comparison of growth curves between *P. salmonis* 5692, 5892 and 5896 (A-E), when the bacteria had starved and been cultivated in different alterations in access to light (figure made by Julia Tandberg). The growth curve of *P. salmonis* 5896 from seawater is not shown because no growth could be detected. In A: The green graph coincides with the purple graph (n= 1).

Sometimes it is of relevance to relate OD to actual cell numbers or- colony forming units for each OD (Fig. 4.3.4). For example, it can be important to know the approximate amount of bacteria added to cells or injected into fish in infection studies. According to Fig. 4.3.4, OD corresponds to approximately the same CFU for the different *P. salmonis* strains.

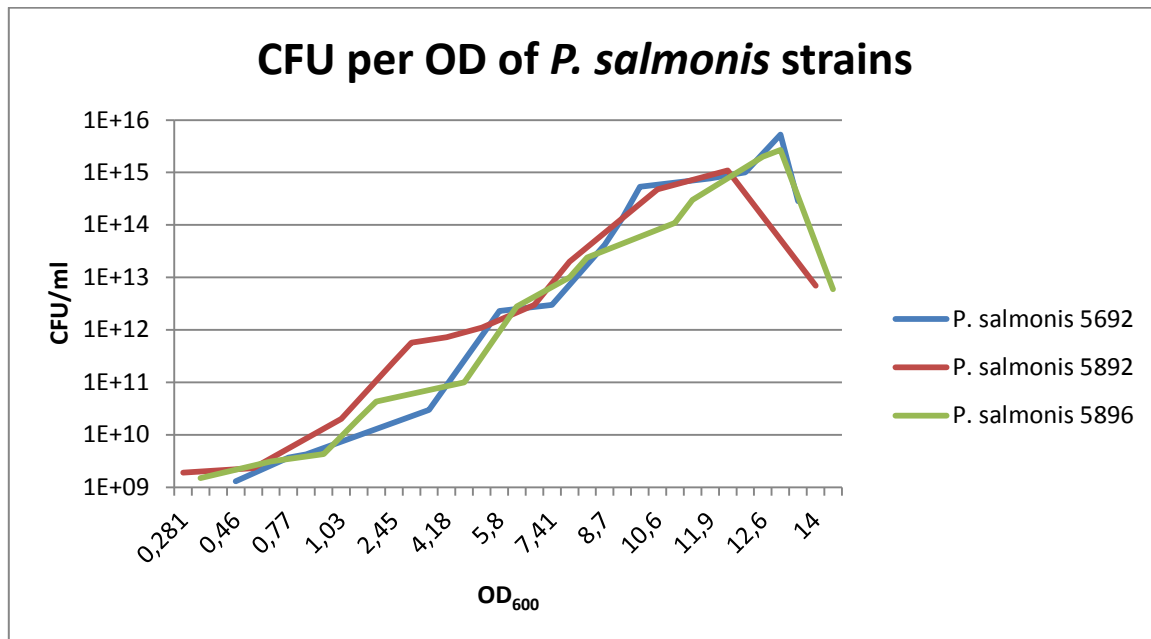


Fig. 4.3.4: The figure shows the relationship between CFU/ml and OD for *P. salmonis* 5692, 5892 and 5896. All cultures were maintained in EBFC medium (n= 3).

4.4 Plasmid isolation

One of the goals in the thesis was to make fluorescently GFP-labeled bacteria, which turned out to be a difficult assignment. Different plasmids and methods were used; electroporation, heat-shock transformation and cryo-transformation, without any success, even though the method conditions were changed. Therefore, plasmid isolation was performed to test whether *P. salmonis* have naturally occurring plasmid(s) which could be an explanation for the difficulties in transforming *P. salmonis* with broad spectrum plasmid vectors known to replicate in at least 41 other Gram-negative bacterial species (Blatny et al., 1997, Thomas and Helinski, 1989). Recently the genome sequence for *P. salmonis* LF-89 was published, but without report of any plasmid in the sequence. However, after isolation of the plasmids from all three *P. salmonis* strains, they were visualized on a 1% agarose gel. The gel clearly showed separate bands for each strain (Fig. 4.4.1), which indicates that *P. salmonis* have

naturally occurring plasmids. There are little differences in the plasmid profile between the strains, and the sizes of the plasmids are at least over 10 kB compared to the DNA ladder.

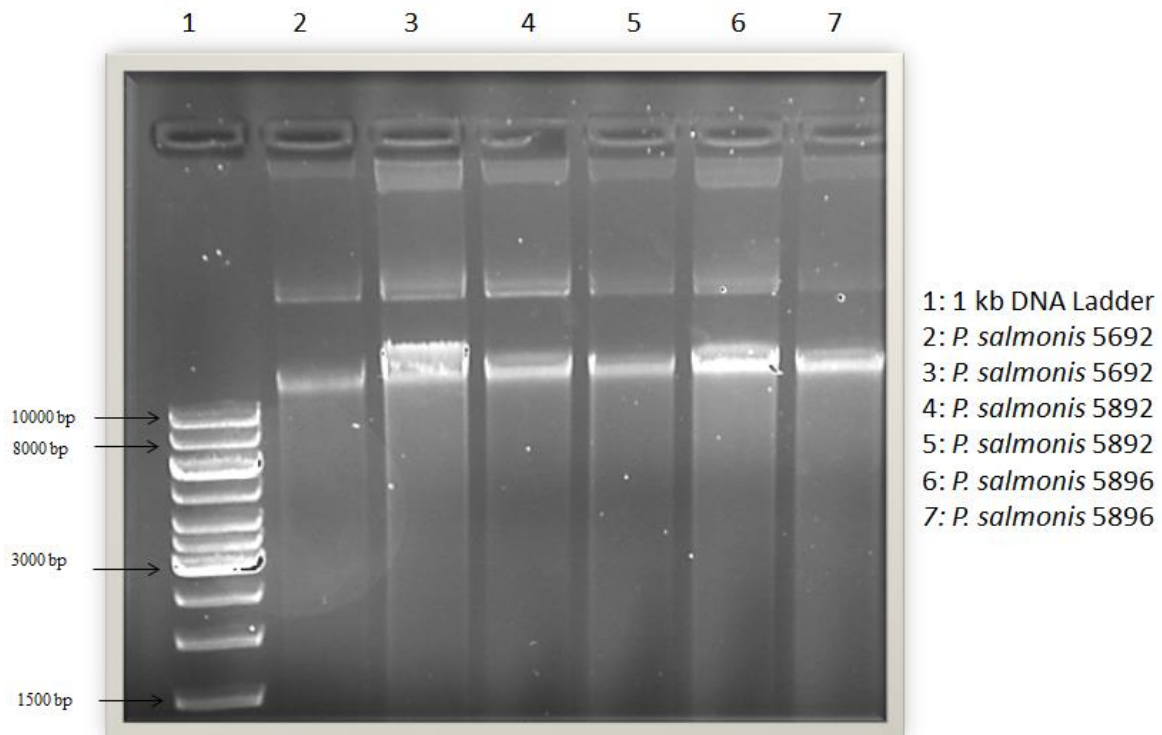


Fig. 4.4.1: Agarose gel electrophoresis of naturally occurring plasmids isolated from *P. salmonis* strain 5692, 5892 and 5896. Well 2 and 3: Plasmids isolated from *P. salmonis* 5692. Well 4 and 5: Plasmids isolated from *P. salmonis* 5892. Well 6 and 7: Plasmids isolated from *P. salmonis* 5896.

4.5 Electron microscopy

Due to the successful establishment of growing *P. salmonis* strains in EBFC liquid medium, it was of natural interest to investigate the shape and size of the bacteria in the electron microscope to investigate any potential phenotypic difference between the strains. Analysis of phenotypic structures was first performed in TEM (Fig. 4.5.1). Different methods were tested; preparation directly from a liquid culture, fixation in liquid culture before preparation or directly from plate. All methods led to bacterial collapsing, and the images where the bacteria were prepared directly from plates; had too much noise. However, fixation in liquid culture before preparation gave best results, due to less collapsing and noise. Only the image of *P. salmonis* 5896 (fixed directly into the liquid culture) is shown as there was no apparent difference between the strains. By TEM analysis they all appeared round in shape with indications of a rippled membrane. However, many of the bacteria had collapsed and lost their

structure. This collapse might be caused by the sensitivity to uranyl acetate (UA) that was used as a negative stain in the preparation of the bacteria, or by loss of bacterial integrity in the buffered solution. Thus, only 1.5 % UA was used in the preparations and the grids were incubated for only one second. In addition, methyl-cellulose with UA was tried to avoid the collapsing unfortunately without too much success (Fig. 4.5.1).

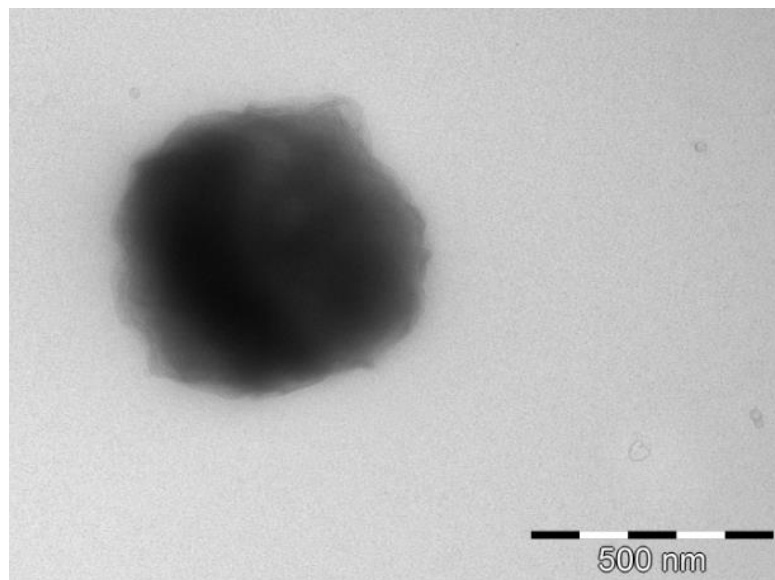


Fig. 4.5.1: TEM analysis of *P. salmonis* 5896

The cell surface of *P. salmonis* was also studied by SEM. Two preparation methods were used in SEM: preparation directly from a liquid culture and directly from plate. In the images where the bacteria were prepared directly from plates detailed cell surface analysis was inhibited by the secretion of bacterial extracellular material. However, it was possible to see signs of the formation of a biofilm of *P. salmonis* (Fig. 4.5.2 D). The best result was obtained when the bacteria were taken directly from a liquid culture and incubated on coverslips. The pictures clearly show that *P. salmonis* have a strong, ruffled membrane; they are coccoid and interestingly structure that bare resemblance to vesicles formation (Fig. 4.5.2 A-C). The observation of these outer membrane vesicles (OMVs) led to further studies.

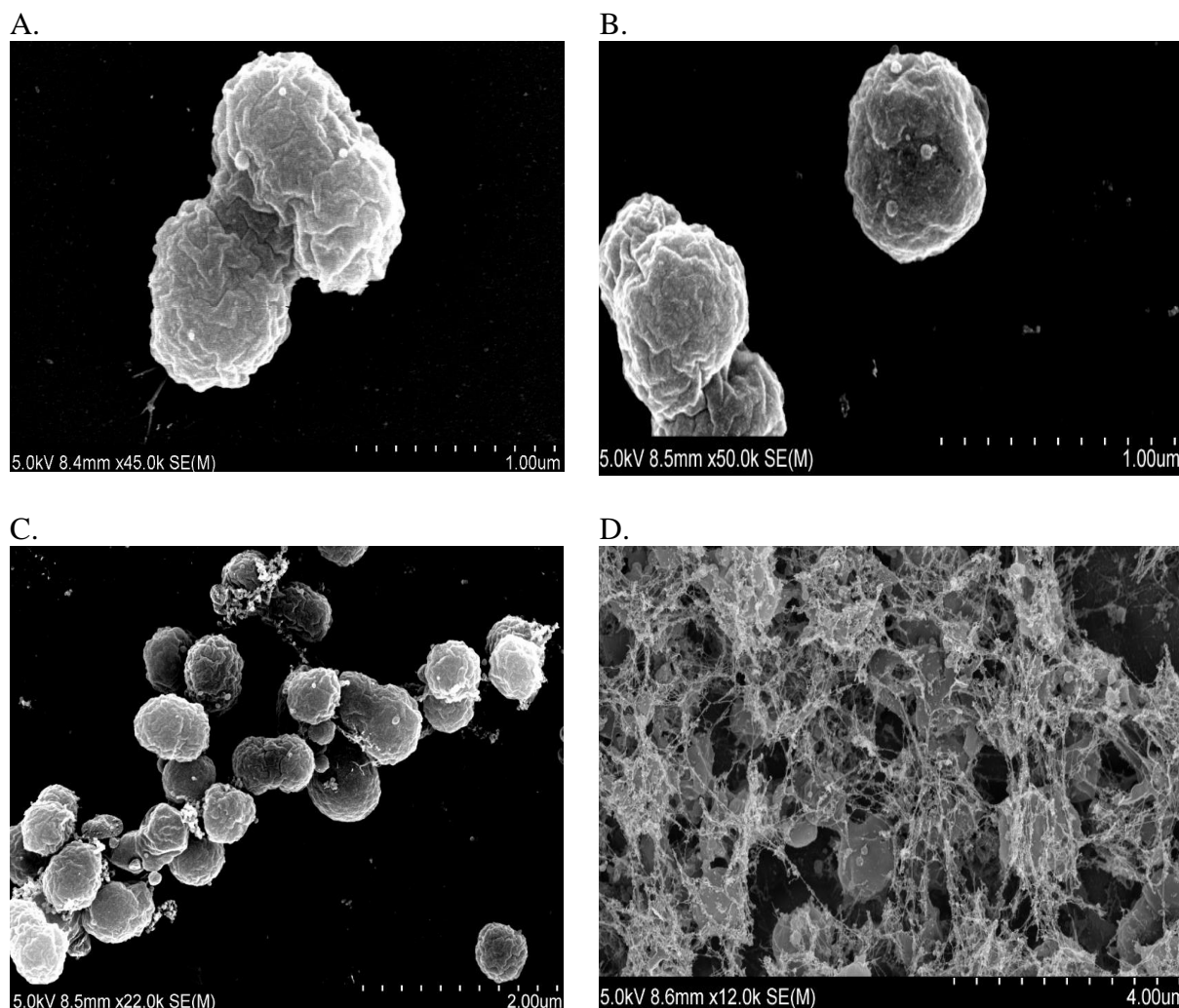


Fig. 4.5.2: SEM images of the cell surface structure of *P. salmonis*. A: *P. salmonis* 5692 from liquid culture. B and C: *P. salmonis* 5892 from liquid culture. D: *P. salmonis* 5896 from agar plate.

4.6 Analysis of outer membrane vesicles from *P. salmonis*

After the observation by SEM that *P. salmonis* secretes membrane vesicles, they were attempted isolated by standard protocols. Subsequent isolated OMVs had a yield of 3.5-4 mg/ml from *P. salmonis* 5692 and 5896, and 2.0 mg/ml for *P. salmonis* 5892. After the isolation; they were analyzed by TEM, to verify the size and morphology (Fig. 4.6.1). The OMVs appeared the same for all three *P. salmonis* strains; numerous, intact and spherical shape at different sizes_ between 20-200 nm.

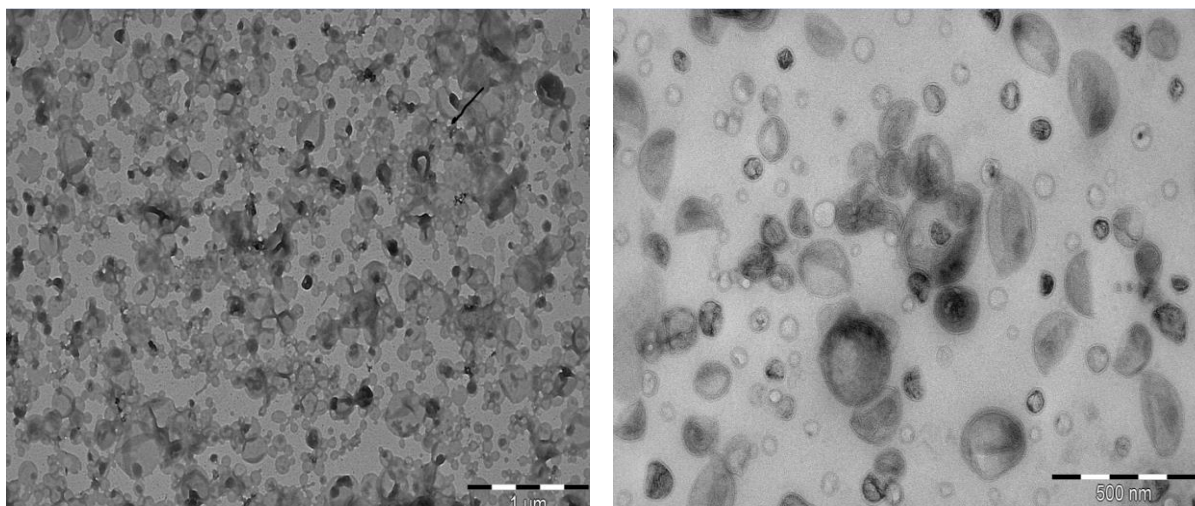


Fig. 4.6.1: TEM images of OMVs isolated from *P. salmonis* 5896. Left image: An overview of the vesicles. Right image: The size and shape of the vesicles.

4.7 Protein content analysis of outer membrane vesicles

Further analyses of the OMVs were performed by SDS-PAGE and Western blotting. Several abundant proteins of different sizes from the OMVs were detectable by Coomassie Blue staining after separation by SDS-PAGE (Fig. 4.7.1). The protein pattern was roughly the same from all *P. salmonis* strains, and in addition the same pattern was confirmed with Western blotting. The gel picture from Western blotting is not shown due to poor quality and resolution of the image.

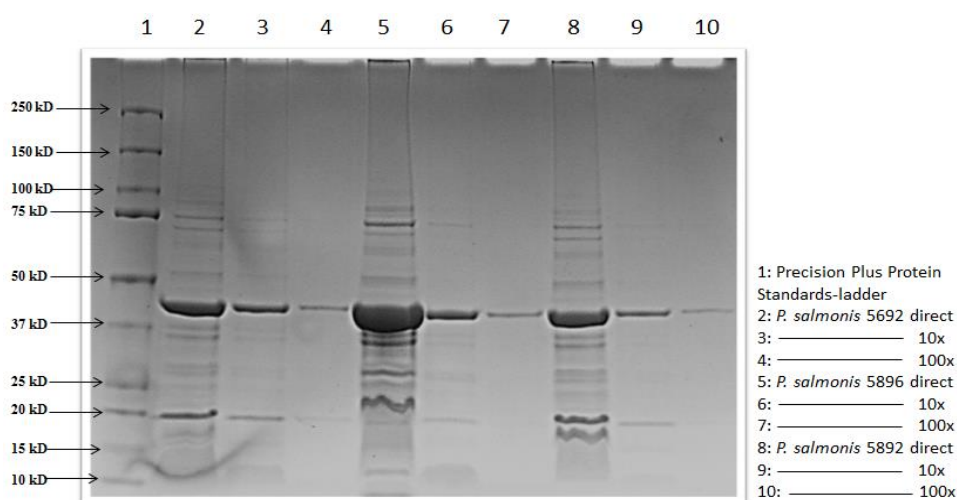
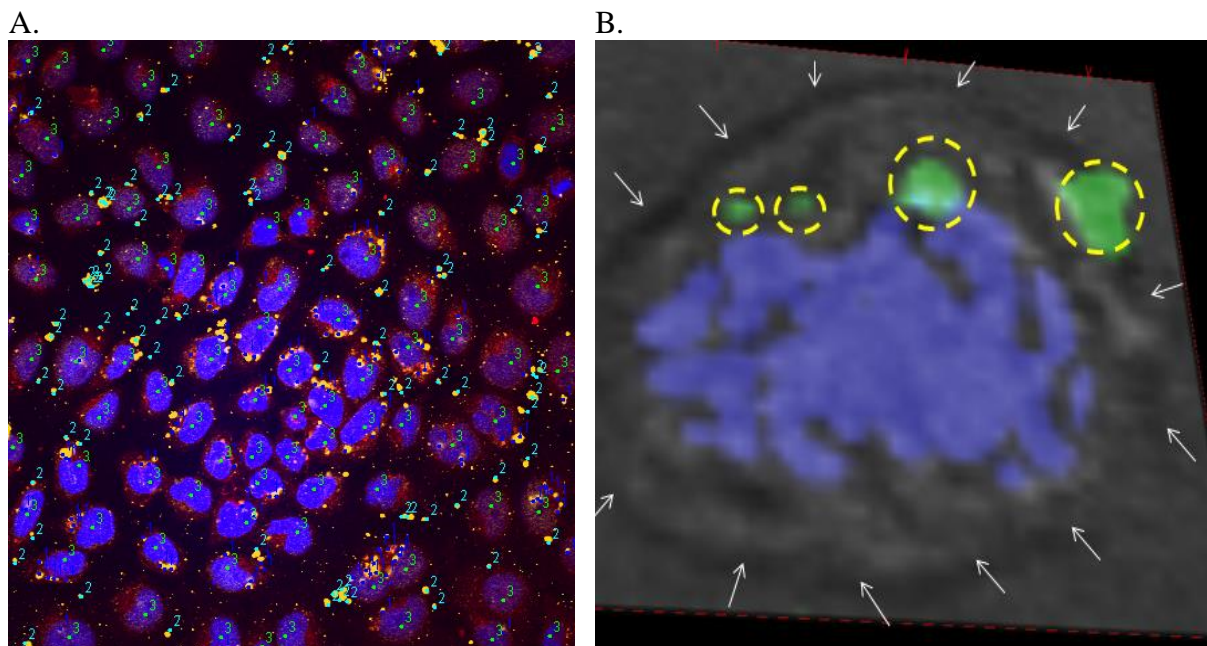


Fig. 4.7.1: Analysis of the protein content in the outer membrane vesicles by Coomassie Blue staining after separation by SDS-PAGE.

4.8 Infection of CHSE-214 cells

According to the literature (Fryer et al., 1992), *P. salmonis* can infect CHSE-214 cells. This is also true for *P. salmonis* strain 5692, 5892 or 5896 as tested here (Fig. 4.8.1). Previous experiment in this study has shown that *P. salmonis* thrives in the dark; therefore it was of interest to investigate if it was a difference in infection of CHSE-214 cells in light and dark. Manually counting of cells and bacteria inside and outside of the cells were performed. This will give an estimation of the infection properties of the bacteria; the more bacteria that are inside the cells, the greater is the infection. The infection in light and dark can then be followed, and whether *P. salmonis* thrives in the dark *in vivo* can also be tested. The counting was possible due to different staining of the CHSE-214 nucleus (blue), the membrane (red) and the bacteria (yellow/green). The bacteria were easily separable from dust and auto fluorescence, due to the larger and reproducible size of the bacteria. The bacteria inside and outside could then be counted, in addition to the number of CHSE-214 cells (Fig. 4.8.1 A). Furthermore, a graph was made to see if there was a striking difference in infection in light and dark (Fig. 4.8.1 C). According to the graph, *P. salmonis* 5892 and 5896 have around 10-15 % higher infection in the dark. In addition, 3D images were taken to confirm that the bacteria were in fact inside the cells, and not on the top or below the cells. Fig 4.8.1 B, clearly illustrate that the bacteria were inside the cells. All of these results were obtained at day 5 post-infection.



***P. salmonis* infection in CHSE cells, day 5, OD= 0.6**

	P. salmonis 5692	P. salmonis 5892	P. salmonis 5896	Control, PBS	P. salmonis 5692	P. salmonis 5892	P. salmonis 5896	Control, PBS
% intracellular	73	64	57	0	73	78	68	0
% extracellular	27	36	43	0	27	22	32	0

Light **Dark**

4.9 Zebrafish embryo survival test

39

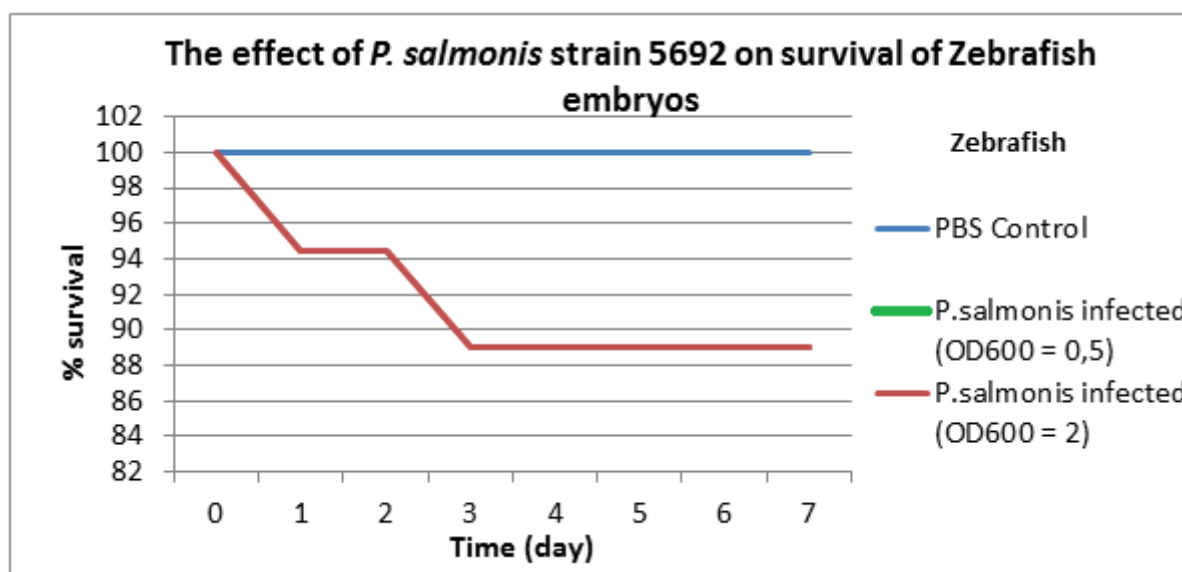
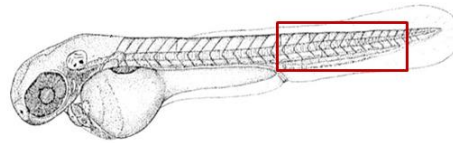


Fig. 4.9.1: Survival experiment of wild type strain AB zebrafish embryos infected with *P. salmonis* 5692 at an OD₆₀₀ of 0.5 and 2 by microinjection. Control embryos were injected with PBS. Each group consisted of 20 embryos, and the embryos were observed daily for mortalities (n= 2).

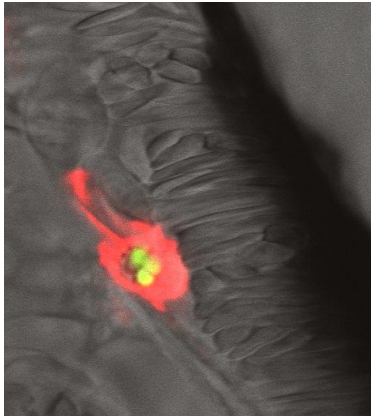
4.10 Infection of Calcein AM-stained *P. salmonis* 5692

As the experiments to genetically transform *P. salmonis* strains with GFP plasmid failed, other methods to follow and visualize *P. salmonis* infections were tried. Calcein AM is a stain that rapidly enters viable cells and thereby gets hydrolyzed inside the cytoplasm where a part of the molecule is removed; it, has previously been used for example to stain retinal ganglion cells (Grieshaber et al., 2010). Calcein AM-stained *P. salmonis* 5692 was injected into 10 zebrafish embryos of Tg(LysC:DsRED2) (red fluorescent protein expressing neutrophils) or into Tg(mpeg1:gal4ff):(UAS:nfsB-mCherry) (red macrophages). After 1 hour the embryos were viewed in the confocal microscope in real-time (Fig. 4.10.1). Tg(mpeg1:gal4ff):(UAS:nfsB-mCherry) embryos with red macrophages had phagocytosed green labeled bacteria. In addition, it was observed that the bacteria attracted more macrophages in the blood stream. On the other hand, Tg(LysC:DsRED2) with red neutrophils; no or little interactions were observed between the neutrophils and the injected bacteria (data not shown).

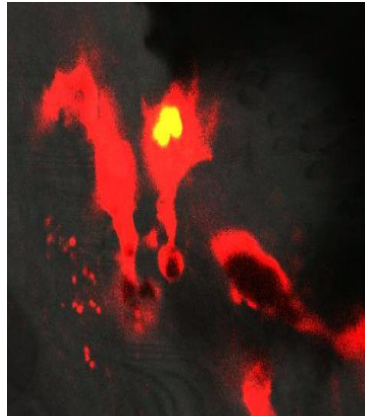
A.



B.



C.



D.

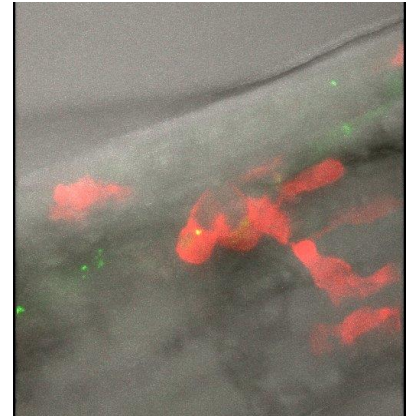


Fig. 4.10.1: All the imaging has been done in the trunk area of the zebrafish embryo (A), specifically in the caudal hematopoietic tissue (CHT). B-D: Red fluorescent macrophages in the zebrafish that have taken up the green calcein AM-stained *P. salmonis* 5692. Images taken by Lilia Ulanova in the confocal microscope.

In the establishment of a zebrafish infection model it is of importance to investigate the immune response of the host to the infection. Thus the immune response from the same fish lines used in imaging was investigated by RT-qPCR by methods previously established by the group for *Francisella* subspecies (Brudal et al., 2014). First the immune response was analyzed for the infection of the Tg(LysC:DsRED2) fish line injected with calcein AM-stained *P. salmonis* 5692. Surprisingly most immune genes were downregulated in the infected fish compared to the PBS control (see appendix C: Other results). Therefore, Tg(mpeg1:gal4ff):(UAS:nfsB-mCherry) embryos injected with calcein AM-stained and non-stained *P. salmonis* 5692 was analyzed to determine if calcein AM itself had an effect on the immune response (see appendix C: Other results). The results obtained indicate that the immune response may be virtually non-existent and calcein AM may have an effect. However, based on the data obtained it is not possible to draw any conclusions and the experiment will have to be repeated.

4.11 Whole-mount immunohistochemistry

Another method to investigate the intracellular localization of *P. salmonis* in the zebrafish is by whole-mount immunohistochemistry. The casper (*roy*^{-/-};*nacre*^{-/-}) zebrafish embryos were

injected with *P. salmonis* 5692, 5892 or 5896 and PBS. At day 0, 1, 3 and 6 post infections, the embryos were euthanized and analyzed with whole-mount immunohistochemistry. By this method the embryos were made fluorescent in blue with DAPI and the bacteria were fluorescently red using immunofluorescent antibodies. Only the result of one fish infected with *P. salmonis* 5692 is presented (Fig. 4.11.1), because there was no apparent difference between the *P. salmonis* strains. The only difference to be detected was between the different days when the embryos were euthanized. Of the several parallel fish from each day they all showed the same results; it took some time for the bacteria to grow inside the fish, and it was not until day 6 post infection that the bacteria had grown to an extent that allowed detection inside the fish. To verify that the bacteria were indeed inside the fish, confocal images were taken (Fig. 4.11.1 E). This picture confirmed the *in vivo* localization of the bacteria inside the fish, and it showed that one single fluorescent signal actually was aggregates of 10-15 bacteria. In contrast, the PBS control injected fish were free of bacteria.

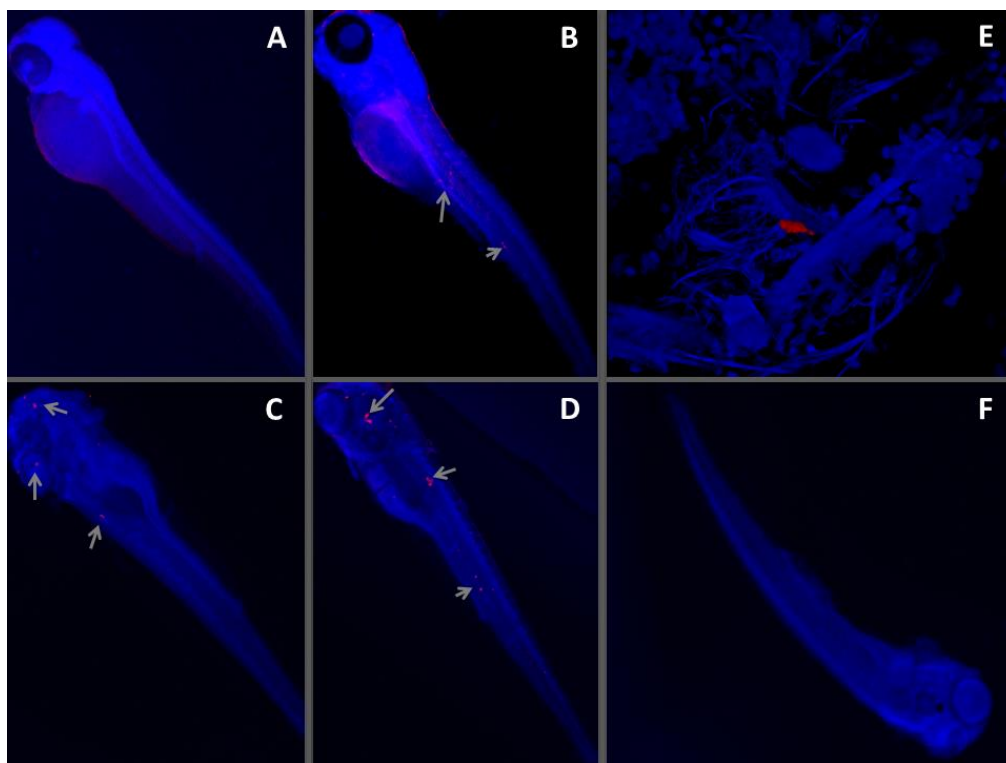


Fig. 4.11.1: *P. salmonis* 5692 infection of zebrafish embryo analyzed by a fluorescence or confocal microscope. A: Embryo at 0 day post infection. No indication of bacterial infection is evident. B: Embryo at 1 day post infection. Some indication of red fluorescently labeled bacteria is evident (arrows). C: Embryo at 3 day post infection. The red bacteria are marked with arrows. D: Embryo at 6 day post infection. The red bacterial clumps are inside the fish (arrows). E: Confocal image of the infected fish at day 6 post infection. The muscles are on the sides of the spinal cord and are adjacent to the point where the red bacteria are located. The red fluorescent signal consists of 8-10 bacteria in one aggregate. F: Control fish injected with PBS at day 6 post infection; no red fluorescently labeled bacteria are visible.

5 Discussion

The basis of *P. salmonis* infection and pathogenesis is not known, and further research on the bacterium is needed. Thus, the scientific field on *P. salmonis* has expanded, due to the spreading, deleterious disease caused by the bacterium. This Master's thesis describes the study on the fish pathogen *P. salmonis*; its growth and investigation to its pathogenesis. This was achieved using the advantages of the zebrafish model system and growth was tested using standard microbiology methods.

5.1 Identification of *P. salmonis* by 16S rRNA sequencing and its growth conditions

There are many reasons why the 16S rRNA gene sequences can be used to study bacterial phylogeny and taxonomy; it is present in all bacteria, the function of the 16S rRNA gene has not changed over time, the 16S rRNA gene is large enough for informatics purposes and the method is easy and economical to perform (Janda and Abbott, 2007). Additionally, the primary structure of the 16S rRNA is highly conserved, and species that have 70 % or greater DNA similarities usually have more than 97 % sequence identity (Stackebrandt and Goebel, 1994). Therefore, if two sequences have a similarity above 97 %; it can be considered that they belong to the same species. However, even this high sequence similarity may not be sufficient in all instances to guarantee an accurate identification. Janda and Abbott (2007) demonstrated that even though the type strains of *Edwardsiella* species exhibit 99.35 to 99.81 % similarity to each other; they are still clearly distinguishable biochemically and by DNA homology (Janda and Abbott, 2007). Many other genomic regions have also been used to identify the phylogenetic relationships among bacteria (Clarridge, 2004) such as whole-genome analysis, 16S-23S rRNA gene internal transcribed spacer sequences and 23S rRNA sequences. 16S rRNA sequencing is still the most preferable method, and this method was used in the thesis and previously in the literature (Rozas and Enríquez, 2013) to identify *P. salmonis*. Once *P. salmonis* 5692, 5892 and 5896 were confirmed to be *P. salmonis* by 16S rRNA sequencing, further studies were undertaken. The quality of obtained sequences allowed identification of the bacteria, and *P. salmonis* 5692 was identified as *P. salmonis* NVI 5692 with a 98 % identity. After this confirmation, growth studies were performed.

Optimal growth conditions for *P. salmonis* were found by testing the bacterial cultures at different temperatures and culture mediums. Mikalsen et al. (2008) were the first to report the ability of *P. salmonis* to grow on agar plates supplemented with 5 % ovine blood (CHAB) (Mikalsen et al., 2008). *P. salmonis* were shown to have an optimal growth at 22 °C, and the colonies reached 1 mm in diameter by day 6. However, present study demonstrated that *P. salmonis* grew faster on ECA plates at 20 °C, and colony size measurement was impossible on day 6 as the colonies growth was too dense. Thus better conditions for *P. salmonis* growth were found as compared to previously reported. There are other reports regarding the optimal growth medium for *P. salmonis* (Yanez et al., 2013, Henriquez et al., 2013). Henriquez et al. (2013) demonstrated growth of *P. salmonis* LF-89 up to a maximum OD₆₀₀ value of 1.7 only within 37.5 hours on a blood-free bacteriological medium with yeast extract and peptone. This growth rate is 3 times faster than those reported previously. The *P. salmonis* strains tested in this study could grow to an OD₆₀₀ value of 12-16 in the EBFC medium within 40-72 hours. Whether the type strain *P. salmonis* LF-89 also can grow in this medium or why the strains tested in the thesis could grow to such high OD₆₀₀ values is not known. The three *P. salmonis* strains tested in this study were found not to grow up to high OD₆₀₀ values in the EBFC medium without casamino acids (EBF medium) even though it was suitable for another fish pathogen *Francisella* (Brudal et al., 2013). Perhaps the casamino acid mixture contains essential amino acids or a specific ingredient, which in combination with iron, makes the environment favorable for the bacteria. At least, it is known that iron is essential for almost all living organisms because it is involved in a wide variety of important metabolic processes. Iron is not available for bacteria and therefore they need to employ various iron uptakes from their surroundings (Wooldridge and Williams, 1993). Most intracellular bacteria need iron, and of course, amino acids. The casamino acids mixture can provide most of the essential nutrients, and it has been used previously to culture bacteria such as *Lactobacillus* (Van Niel and Hahn-Hägerdal, 1999). However, further studies are needed to find the specific ingredient of casamino acids that is essential for *P. salmonis* growth. This can be further investigated by successively removing one of the ingredients of the casamino acid mixture which will reveal the amino acid(s) that is the most important for bacterial growth. Additionally, no antibiotics were used in the medium due to the sensitivity of *P. salmonis* to most of the antibiotics tested in the thesis. Therefore creating a bacterial strain with an antibiotic resistance would be one of the appealing directions to allow growing *P. salmonis* selectively.

5.2 Antibiotic susceptibility

One of the most important routines for microbiological laboratories is the performance of antibiotic susceptibility testing on bacterial isolates. There are several methods available to do this testing such as broth dilution test, antimicrobial gradient test, disc-diffusion test or with automated instruments (Reller et al., 2009). The disc-diffusion test was chosen in the thesis because the method has previously been used to test *P. salmonis* antibiotic sensitivity in the literature (Mikalsen et al., 2008). The disc-diffusion method is a simple test that does not require any special equipment, it is a low-cost method and one can be flexible in selection of discs for testing. However, the test cannot be automatized and not all fastidious or slow growing bacteria can be accurately tested by this method (Reller et al., 2009). The results obtained after the performance of a disc-diffusion method indicated that *P. salmonis* are sensitive to a variety of antibiotics *in vitro*, in agreement with data reported earlier (Mikalsen et al., 2008, Fryer et al., 1992). In this thesis, *P. salmonis* appeared to be resistant only to sulfadiazine and sulfamethoxazole out of 13 antibiotics that have been tested. Even though the bacterium is sensitive to antibiotics *in vitro*, the situation is not always the same *in vivo* (Rozas and Enríquez, 2013). This is most likely due to the fact that the bacterium is intracellular, and perhaps insufficient concentration of antibiotics reaches the bacteria within the host. Therefore, *P. salmonis* should be grown in the absence of antibiotics as their presence can affect the results obtained during growth measurements.

5.3 Growth curves and CFU

P. salmonis 5692, 5892 and 5896 tested in the thesis had similar growth curves (Fig. 4.3.1) with *P. salmonis* 5892 being the slowest growing strain. When the bacteria reached their optimal OD₆₀₀ for inoculation, the cultures were diluted to an OD₆₀₀ 0.02 and left to grow. This is followed by so-called lag phase, the time required by cells to synthesize and to accumulate metabolite(s) that are essential for growth and division (Lankford et al., 1966). Therefore, the length of this phase depends directly on the growth phase of the inoculum. Additionally, Augustin et al. (2000) demonstrated that the duration of the lag phase for *Listeria monocytogenes* depends strongly on the size of the inoculum (Augustin et al., 2000). The lag time increased with a decreased inoculum size of previously starved cells. The different *P. salmonis* strains tested in the thesis, also had lag phase duration dependent on the size of the inoculum. *P. salmonis* 5892 needed a high inoculum to decrease the lag phase,

while *P. salmonis* 5892 and 5896 needed a lower inoculum. The average lag phase for the three *P. salmonis* strains are approximately from 0-25 hours with an OD₆₀₀ of 0-1. According to Fig. 4.3.3 an OD₆₀₀ of 1 correspond to approximately 10¹⁰ CFU/ml for each *P. salmonis* strain. The lag phase is followed by rapid bacterial growth called exponential phase. For *P. salmonis*, this phase lasts approximately from 26-47 hours. When all the essential nutrients in the growth medium are used up and the reproduction rate is slow or non-existent, the bacteria enter the stationary phase. This phase happens after 48-72 hours with some variations within the different *P. salmonis* strains. Eventually the bacteria proceed to the death or logarithmic decline phase. After approximately 72 hours, all of the three *P. salmonis* strains tested in this study were in this phase. Additionally, liquid cultures of the different *P. salmonis* strains with the same OD₆₀₀ had almost the same CFU/ml ratio during the whole growth process.

The following experiment was initiated due to an interesting observation that *P. salmonis* grows faster during the night with no light exposure. The bacterial growth after starvation in seawater was observed with and without light exposure in EBFC medium. As shown in Fig. 4.3.2 *P. salmonis* could survive in seawater for a period of time, and in this condition they grew more when exposed to light. However, after the transfer from the seawater to the rich EBFC medium, the bacteria grew faster in the dark. Once proven correct by repetition, this result can initiate further studies to find the biological reason behind this phenomenon. This finding could imply a circadian rhythm of *P. salmonis*, even though the bacterium is non-photosynthetic. Soriano et al. (2010) reported an heterotrophic bacterium *Pseudomonas putida*, that showed regular variations in its growth pattern synchronized with light/darkness cycles (Soriano et al., 2010), which could be an advantage in certain environments. The authors cultured the bacterium on agar medium containing either Coomassie brilliant blue or Congo red. These dyes bind proteins and other extracellular polymeric substances; allows direct visualization of surface changes appearing during colony growth in light and dark. This experiment was repeated in the thesis; however, no difference in growth pattern in light and darkness has been indicated (results not shown).

Another possible theory is that the light and dark cycle of *P. salmonis* is caused by membrane-embedded, light-driven proton pumps called proteorhodopsins. These pumps translocate protons across the membrane, generating available energy in the form of adenosine triphosphate (ATP) (Gómez-Consarnau et al., 2010). Gómez-Consarnau et al. (2010) showed that marine *Vibrio* species that contain proteorhodopsins, display light-

enhanced survival during starvation in seawater. Furthermore, bacteria that have been starved in the light could respond more rapidly to improved growth conditions faster than those that have been incubated in the dark. Gómez-Consarnau et al. (2010) investigated this phenomenon by creating proteorhodopsins deficient *Vibrio* strain and managed to demonstrate that the light-dependent survival after starvation were in fact mediated by the proteorhodopsins. Whether this is the case for *P. salmonis* remains unclear, and further studies are needed to confirm the light and dark cycle.

5.4 Plasmid isolation

Plasmids are small, typically circular, double-stranded DNA molecules that are distinct from the cellular chromosomal DNA and can exist in three conformations: supercoiled, open-circular and linear (Jong et al., 1997). There are no reports of *P. salmonis* plasmids in the literature. It was only recently that- the first draft genome sequence of *P. salmonis* LF-89 was published (Eppinger et al., 2013).

Plasmid isolation was performed due to problems with making fluorescently GFP-labeled *P. salmonis*. Different transformation methods such as electroporation, heat-shock and cry-transformation were used, in addition to different vector plasmids, without any success (results not shown). The results obtained after the plasmid isolation clearly shows that *P. salmonis* have naturally occurring plasmids (Fig. 4.4.1). Naturally occurring plasmids can sometimes block the replication of the vector plasmid if the vector plasmid is related genetically to the naturally occurring plasmid- incompatible plasmids (Velappan et al., 2007). Even though, a method called plasmid curing with ethidium bromide was performed in the thesis to eliminate the naturally occurring plasmid, it was unsuccessful (results not shown). Another possible reason for the transformation problem is that the vector plasmid may need a *P. salmonis* promoter to be able to recognize and replicate the plasmid, and thereby expressing the green-fluorescent insert in the vector plasmid. To obtain this, the naturally occurring plasmid needs to be sequenced, which is an ongoing experiment. The gel capture (Fig. 4.4.1) shows little differences in plasmids composition between the strains, and the sizes of all the plasmids are at least over 10 kB. The plasmids are most likely low-copy number, and it remains unclear whether there are several plasmids of the same size, plasmids of different sizes or only one plasmid. What genes the genome and the plasmid contain is not certain, or whether there is a difference between the strains. The purpose the plasmids serves

in *P. salmonis* is not known, but usually the plasmids give a genetic advantage for the bacteria, such as a unique metabolism or antibiotic resistance. The *P. salmonis* strains tested in the thesis were quite similar in regard to antibiotic sensitivity, growth conditions and plasmids. Therefore, it was also interesting to test whether the different bacteria strains were similar in size and shape.

5.5 Size and shape of *P. salmonis* and OMVs

The size and shape of *P. salmonis* is widely reported to be coccoid with a size of 0.5-1.5 μm (Fryer et al., 1990, Fryer et al., 1992, Fryer and Hedrick, 2003). The present study using SEM and TEM confirmed that the bacteria are coccoid with indications of a rippled membrane. There was no apparent difference in size or shape between the studied strains. In addition, observations by SEM revealed other biological structures, such as a biofilm and vesicles. Biofilms are formed when bacteria are organized into a structure on solid surface and surround themselves with exopolysaccharide matrix (Costerton et al., 1999). The biofilm formation by *P. salmonis* may be an response to stress as it has been reported in the literature, e.g. *P. salmonis* produce biofilm to cope with stress and survive in marine waters (Marshall et al., 2012).

Outer membrane vesicles (OMVs) are normally produced during the bacteria life span and used for the communication between different individuals, which most likely plays an important role in *P. salmonis* pathogenesis. Outer membrane vesicles are spherical lipid bilayer blebs that are formed by budding from the outer bacterial membrane and contain lipopolysaccharides (LPS) and periplasmic proteins (Kulp and Kuehn, 2010). OMVs are usually 10-300 nm in diameter. Previously, OMVs were thought to be depleted in inner membrane and cytoplasmic components (Katsui et al., 1982), but a recent report indicate that they might contain cytoplasmic proteins and inner membrane in along with DNA (Perez-Cruz et al., 2013). Some pathogenic bacteria load their OMVs with virulence factors e.g adhesins and toxins, and these vesicles can then be delivered to distant host cells (Kuehn and Kesty, 2005). Since the OMVs in their native state contain surface-exposed antigens e.g. porins or LPS (pathogen-associated molecular patterns), they are able to stimulate Toll-like receptors on the surface of phagocytes and thus trigger immune response (Collins, 2011). OMVs appear to be safe as vaccines, and they have previously been given to humans infected with *Neisseria meningitidis* type B with great success (Collins, 2011). It has in addition been shown that *P.*

salmonis can produce OMVs in cultured rainbow trout head kidney macrophages (McCarthy et al., 2008).

The OMVs produced by the bacteria in liquid culture were isolated and analyzed in the thesis. The obtained yield varied between the different strains. The isolated OMVs from *P. salmonis* 5692 and 5896 had a yield of 3.5-4 mg/ml, and *P. salmonis* 5892 had around 2.0 mg/ml. The lower yield for *P. salmonis* 5892 is probably due to slower growth rate of this strain compared to the other two. Subsequently, the size and shape of OMVs was analyzed by TEM. The OMVs observed by TEM were numerous, intact, spherical, and at different sizes between 20-200 nm. There are no reports on OMVs protein composition and their function.

However, the protein composition and function in the OMVs from other pathogens has been studied more extensively. Pierson et al. (2010) performed a proteomic analysis of the OMVs from a related fish pathogen *Francisella*. The authors demonstrated several proteins involved in pathogenesis and secretion system such as PdpA which is known to be delivered from *Francisella* to target host cells. In addition, beta lactamase were found in the OMVs, and its presence could help the bacterium to alter the antibiotic concentration in their external environment (Pierson et al., 2010). Since *Francisella* and *P. salmonis* are closely related, it may be that their OMVs also share similarities in regards to protein composition and may be their functions. In the thesis, SDS-PAGE and Western blotting showed that the OMVs produced by *P. salmonis* contained several abundant proteins of different sizes from 15 kDa to 100 kDa. The most abundant protein was around 37-50 kDa in size. There are ongoing studies in the Winther-Larsen lab to identify the OMVs protein content.

5.6 Infection of CHSE-214 cells

In the thesis the results obtained by Fryer et al. (1992) were confirmed, showing that *P. salmonis* are able to infect, survive and propagate inside CHSE-214 cells (Fryer et al., 1992). Due to the interesting growth effects seen under light and darkness conditions in this thesis, infection of CHSE-214 cells were also tested in light and in darkness. There are no reports in the literature regarding light and darkness infection of *P. salmonis* in fish cells. The cell count was performed on multiple cells and the results of the counting showed that *P. salmonis* tend to have around 10-15 % higher infection in the dark. Whether there are a statistical significant difference between infection in light and dark was not tested due to lack of time. Repetition of

the experiments is required to support these preliminary data. In addition, the bacteria were confirmed to be localized inside the cells by confocal imaging using Z-stack option; by using a conventional wide field fluorescence microscope it was hard to tell whether the bacteria were inside, below or on the surface of the CHSE-214 cells. Due to poor staining of the cell membrane and that the dye also seemed to stain bacterial membranes, the creation of 3D image structures was performed. By 10 days post infection, 80 % of the bacteria were inside the cells although by this time the cells appeared to be in poor condition, presumably due to consequence of the infection (result not shown). This event is called cytopathic effect (CPE) which is typical for *P. salmonis* infection (Olsen et al., 1997). In contrast, the control CHSE-214 cells treated only with PBS were numerous and showed no signs of CPE (result not shown).

CHSE-214 cells were chosen in the thesis due to the fact that this cell line is the most used and accepted model to culture *P. salmonis* LF-89 in the literature (Fryer et al., 1990, Fryer et al., 1992, Fryer and Hedrick, 2003). The first *in vivo* culture of *P. salmonis* was in the CHSE-214 cell line, while there are now several reports in the literature describing *P. salmonis* replications in other fish cell line e.g Atlantic salmon kidney (ASK) cell line (Smith et al., 2014). ASK- cells are more flat than the CHSE-214 cells, and therefore CHSE-214 cells are more convenient to use to check if the bacteria are inside the cells. Even though studying *P. salmonis* pathogenesis using fish cell lines can give many answers concerning pathogenesis, for many issues there is no substitute for using a complete organism.

5.7 Zebrafish embryo survival test

The zebrafish is a well-known model to study pathogenesis of different bacteria due to the fast development and the available transgenic lines. Additionally, the adaptive immune system is not fully functional until 4-6 weeks post fertilization which gives an opportunity to study the innate immune system apart from the adaptive. The model has previously been used with great success for *Mycobacterium marinum* (Prouty et al., 2003) and *Francisella* (Brudal et al., 2014). Using the same protocol established by Brudal et al. (2014), *P. salmonis* infection in the wild type strain AB zebrafish embryos was tested. According to the results in this thesis, *P. salmonis* infection does not cause an acute disease in this fish. An acute disease is characterized by a sudden onset of symptoms that usually are severe. Only two fish died in each experimental group out of a total of 20 fish regardless of the infection dose. No mortality

was observed when the fish were injected with PBS- illustrating that the injection itself was not the reason for deaths among the embryos. It appears that *P. salmonis* causes a chronic infection in the zebrafish embryos, reminiscent of that observed for a related fish pathogen *Francisella noatunensis ssp. noatunensis* (*F.n.n*) (Brudal et al., 2014). Survival experiments by Brudal et al. (2014) demonstrated that *F.n.n* infection did not lead to any significant mortality regardless of the amount of bacteria used for infection. This is most likely due to the extremely slow growth rate of this bacterium. The immune response results indicated that *F.n.n* causes a chronic infection in the zebrafish embryos. Whether this is the case for *P. salmonis* infection, remains to be shown. There are no reports in the literature as to whether *P. salmonis* is able of infect, survive and propagate in zebrafish host. In this thesis the *P. salmonis*-zebrafish embryo infection model was established. A recent pilot study done by Julia Tandberg, confirmed that *P. salmonis* can infect and cause disease in adult zebrafish (unpublished results). Using the different zebrafish lines in combination with fluorescently labeled pathogens allows real-time *in vivo* observations of disease progression. Due to difficulties in making fluorescently-labeled *P. salmonis*, other methods to follow and visualize *P. salmonis* were necessary.

5.8 Infection of Calcein AM-stained *P. salmonis* 5692

A dye called Calcein AM was used to color the *P. salmonis* 5692 membrane. Calcein AM is an intracellular fluorescent marker commonly used for evaluation of cell volume and viability. It is a vital dye that rapidly enters viable cells and then it is hydrolyzed whereby a part of the molecule is removed (Grieshaber et al., 2010). The molecule gets trapped inside the cytoplasm and emits a strong fluorescence in the green part of spectrum with light emission at 400-500 nm. Calcein AM is only retained within cells with an intact plasma membrane while- dying or damaged cells will therefore not display a fluorescent signal, allowing evaluation of viability (Grieshaber et al., 2010). Calcein AM was chosen because it has been tested on other bacteria (Dass et al., 2009) and available “in-house”. This dye stained the *P. salmonis* 5692 membrane, however not all the bacteria were stained. The reason for this is not known, but a possible explanation could be that; the bacteria were dead. A state of the *P. salmonis* membrane structure could also make it impermeable for the dye. In addition, after adding a pulse of calcein AM the stained bacteria lost the fluorescent signal after approximately 3 hours due to division and growth of the bacteria.

Despite these limitations, the early events of injection in zebrafish embryos were imaged. Calcein AM-stained bacteria were injected in the transgenic fish line Tg(mpeg1:gal4ff): (UAS:nfsB-mCherry) with red fluorescent macrophages phagocytosed the green fluorescent bacteria. The co-localization of bacteria and macrophages were observed as early as 1-3 hours post infection. This results is confirmed previously in the literature, showing that *P. salmonis* is phagocytosed by macrophages (McCarthy et al., 2008, Rojas et al., 2010, Rojas et al., 2009). After approximately 3 hours the dye from the bacterial membrane was lost and the bacteria could no longer be observed in the fish. Therefore, the study in this thesis could not yet confirm that the bacteria replicated or escaped from the macrophage, this could only be possible once the bacteria have a permanent fluorescent marker. Brudal et al. (2014) demonstrated the co-localization of macrophage and *Francisella* using the transgenic zebrafish lines expressing red-fluorescent macrophages and the bacteria had a permanent fluorescent marker (Brudal et al., 2014). In this thesis, Tg(LysC: DsRED2) embryos with red fluorescent neutrophils were also injected with calcein-AM labeled *P. salmonis* 5692 showed no significant co-localization with bacteria. This argues that macrophages are the main cells responsible for phagocytosis of injected *P. salmonis* 5692.

5.9 Whole-mount immunohistochemistry

Whole-mount immunohistochemistry is a method to study intracellular localization of *P. salmonis* in the zebrafish post mortal. The method is a good alternative to study *P. salmonis* pathogenesis, but due to the fact that the fish and bacteria are dead makes it impossible to study how dividing bacteria behave inside a live fish. The method was based on the whole-mount immunohistochemistry chapter in Macdonald's book (Macdonald, 1999), and some changes from this method were made in the thesis. The results indicate that *P. salmonis* can infect zebrafish embryos and it is able to grow inside the fish as increasing number of bacteria were observed at later time points. Another method that could be used to detect exactly the amount of bacteria inside a zebrafish macrophage is by flow cytometry. This method presumably would allow using the fluorescent signal from anti-*P. salmonis* antibodies and fluorescent signal in macrophages of transgenic zebrafish lines. Even though the method was established, no results were obtained yet due to the lack of time.

6 Conclusion

The main conclusions that can be drawn from this Master's thesis are:

- The three *P. salmonis* strains 5692, 5892 and 5896 were identified as *P. salmonis* by 16S rRNA sequencing.
- Procedures for growing *P. salmonis* strains in the laboratory were established, giving optimal growth of the strains used.
- *P. salmonis* strains were sensitive *in vitro* to the majority of antibiotics tested, such as kanamycin.
- Optimal OD₆₀₀ of the inoculum that gave the best growth was found and growth curves were made.
- The rate of growth in regard to CFU was approximately the same for all three *P. salmonis* strains.
- *P. salmonis* could survive in seawater for a period of time, and in this period they preferred the light. However, after transferring the bacteria from the starvation period in seawater to EBFC medium, they preferred darkness.
- Transfection of *P. salmonis* with a plasmid containing fluorescent marker appeared not to be feasible.
- *P. salmonis* strains have naturally occurring plasmids.
- The bacteria were confirmed to be coccoid with a rippled membrane, and biofilm- and vesicle formation was observed.
- Isolated OMVs were numerous and spherical with a size of 20-200 nm. They were densely packed with proteins of different sizes.
- *P. salmonis* strains could infect CHSE-214 cells and the infection rate was approximately 10 % higher in the dark.
- A *P. salmonis*-zebrafish embryo infection model was established, and the model was shown to be useful for studies of *P. salmonis* infections.
- Zebrafish macrophages were efficient in phagocytosing the bacteria shortly after infection, whereas neutrophils were barely or not involved.
- Intracellular localization of *P. salmonis* in the zebrafish was confirmed by whole-mount immunohistochemistry and fluorescent imaging.

7 Future perspectives

Little is known about the life cycle of *P. salmonis* and even less data is available regarding its pathogenesis. Therefore future studies should focus on these topics in order to eventually find the cure against piscirickettsiosis.

The pathogen itself was just recently for the first time grown independent of cell lines, which is a significant leap for research on this bacterium. Three *P. salmonis* strains were demonstrated to grow on agar and in liquid medium to high densities in the thesis. Whether other *P. salmonis* strains e.g the type strain LF-89 can grow on the same medium needs to be tested in the future. The study presented in the thesis for the first time revealed light-sensitivity of *P. salmonis*, which brings plenty of new possibilities for studying the bacterial pathogenesis. This was only done once in the thesis and further studies are therefore needed to draw any conclusion.

The study of *P. salmonis* pathogenesis using the zebrafish model would have been much easier if genetically encoded fluorescently-labeled mutant strains of *P. salmonis* were available. Attempts to make *P. salmonis* mutants in the group were until now unsuccessful, and the reason might be due to naturally occurring plasmids within the same plasmid incompatibility group. Sequencing of these plasmids to eventually have fluorescently labeled mutants is an ongoing experiment in the Hanne Winther-Larsen's group (UiO). Infecting the fish with fluorescently labeled bacteria would allow *in vivo* imaging of early events happening after as well as long-term acquisition of the disease progression. In addition, further studies are needed in regard to immune response in fish infected with *P. salmonis*, which appeared to be delayed due to chronic nature of the infection. Therefore studies of the immune response at later time points are required.

Vaccination is the only one available long-term solution intended to eliminate the threat caused by *P. salmonis* in aquaculture. Though, the vaccines that are already on the market show variable long-term efficiency. The market in Chile offers 33 injectable vaccines against piscirickettsiosis, where 29 are inactivated vaccines and 4 are subunit vaccines. There are no vaccines based on outer membrane vesicles produced by *P. salmonis* on the market yet, even though they look appealing. Fortunately, this is an ongoing experiment in the Hanne Winther-Larsen's group and will be tested in both the established zebrafish model and in salmonids in

the near future. Another possible solution is to test whether nanoparticle encapsulation of antibiotics will be a better treatment than free antibiotics. Due to lack of time, this was not tested in the thesis and further studies should focus on testing different encapsulation methods and antibiotics. Further research on this fish pathogen is important, due to the deleterious spreading disease which might develop into an even bigger problem for aquaculture in the future.

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Appendix

A. Solutions:

- **1M HEPES (N-2-hydroxyethylpiperazine-N9-ethanesulfonic acid), pH 6.8 (1L)**

238.3 g HEPES

800 ml MQ-water

Up to 1L MQ-water

Adjust pH

- **PBS (phosphate buffered saline), pH 7.4 (1L)**

8 g Sodium chloride (NaCl)

0.2 g Potassium chloride (KCl)

1.44 g Disodium hydrogen phosphate (Na_2HPO_4)

0.14 g Potassium dihydrogen phosphate (KH_2PO_4)

Up to 1L MQ-water

- **Protein Sample buffer**

6 ml 10 % Sodium dodecyl sulfate (SDS)

3 ml Glycerol

1.9 ml 1M Tris-HCl pH 6.8

0.3 ml 0.2M EDTA

3 ml 50 mM β -Mercaptoethanol

0.8 ml dH₂O

8 mg Bromophenol Blue

- **Coomassie Blue, 100 ml**

45 ml 45 % Methanol

900 μ l 0.9 % Acetic Acid

0.1 g 0.1 % Brilliant Blue R
54 ml MQ-water

- **Destaining buffer, 500 ml**

150 ml 30 % Methanol
50 ml 10 % Acetic acid
300 ml MQ-water

- **0.2M Sodium cacodylate buffer, pH 7.4 (1L)**

4.28 g Sodium cacodylate
1000 ml dH₂O
Adjust pH

- **Embryo water**

1.0 ml Hanks' stock solution #1
0.1 ml Hanks' stock solution #2
1.0 ml Hanks' stock solution #4
95.9 ml H₂O
1.0 ml Hanks' stock solution #5
1.0 ml Hanks' stock solution #6

Adjusted pH to 7.2, filter sterilized and stored indefinitely at 4 °C

- **Hank's stock solutions**

Stock #1: 8.0 g NaCl, 0.4 g KCl, 100 ml H₂O

Stock #2: 0.358 g Na₂HPO₄ anhydrous, 0.6 g KH₂PO₄, 100 ml H₂O

Stock #4: 0.72 g CaCl₂, 50 ml H₂O

Stock #5: 1.23 g MgSO₄·7H₂O, 50 ml H₂O

Stock #6: 0.35 g NaHCO₃, 10 ml H₂O

Long term storage at 4 °C

B. Kit protocols:

• E.Z.N.A.® Gel Extraction Kit - Spin Protocol

1. Perform agarose gel/ethidium bromide electrophoresis to fractionate DNA fragments. Any type or grade of agarose may be used. However, it is strongly recommended that fresh TAE buffer or TBE buffer be used as running buffer. Do not reuse running buffer as its pH will increase and reduce yields.
2. When adequate separation of bands has occurred, carefully excise the DNA fragment of interest using a wide, clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose.
3. Determine the appropriate volume of the gel slice by weighing it in a clean 1.5 mL microcentrifuge tube. Assuming a density of 1 g/mL, the volume of gel is derived as follows: a gel slice of mass 0.3 g will have a volume of 0.3 mL.

4. Add 1 volume Binding Buffer (XP2).

5. Incubate at 60°C for 7 minutes or until the gel has completely melted. Vortex or shake the tube every 2-3 minutes.

Important: Monitor the pH of the Gel/Binding Buffer mixture after the gel has completely dissolved. DNA yields will significantly decrease when the pH > 8.0. If the color of the mixture becomes orange or red, add 5 µL 5M sodium acetate (pH 5.2) to bring the pH down. After this adjustment, the color of the Gel/Binding Buffer mixture should be light yellow.

6. Insert a HiBind® DNA Mini Column in a 2 mL Collection Tube.

7. Add no more than 700 µL DNA/agarose solution from Step 5 to the HiBind® DNA Mini Column.

8. Centrifuge at 10,000 x g for 1 minute at room temperature.

9. Discard the filtrate and reuse collection tube.

10. Repeat Steps 7-9 until all of the sample has been transferred to the column.

11. Add 300 µL Binding Buffer (XP2).

12. Centrifuge at maximum speed ($\geq 13,000 \times g$) for 1 minute at room temperature.

13. Discard the filtrate and reuse collection tube.

14. Add 700 µL SPW Wash Buffer.

Note: SPW Wash Buffer must be diluted with 100% ethanol prior to use. Please see Page 5 for instructions.

15. Centrifuge at maximum speed for 1 minute at room temperature.

16. Discard the filtrate and reuse collection tube.

Optional: Repeat Steps 14-16 for a second SPW Wash Buffer wash step. Perform the second wash step for any salt sensitive downstream applications.

17. Centrifuge the empty HiBind® DNA Mini Column for 2 minutes at maximum speed to dry the column matrix.

Note: It is important to dry the HiBind® DNA Mini Column matrix before elution. Residual ethanol may interfere with downstream applications.

18. Transfer the HiBind® DNA Mini Column to a clean 1.5 mL microcentrifuge tube.

19. Add 30-50 µL Elution Buffer or deionized water directly to the center of the column membrane.

Note: The efficiency of eluting DNA from the HiBind® DNA Mini Column is dependent on pH. If eluting DNA with deionized water, make sure that the pH is around 8.5.

20. Incubate at room temperature for 2 minutes.

21. Centrifuge at maximum speed for 1 minute. Note: This represents approximately 70% of bound DNA. An optional second elution will yield any residual DNA, though at a lower concentration.
22. Store DNA at -20 °C.

• **E.Z.N.A.® Plasmid DNA Mini Kit I Protocol - Spin Protocol**

1. Isolate a single colony from a freshly streaked selective plate, and inoculate a culture of 1-5 mL LB medium containing the appropriate selective antibiotic. Incubate for ~12-16 hours at 37°C with vigorous shaking (~ 300 rpm). Use a 10-20 mL culture tube or a flask with a volume of at least 4 times the volume of the culture. It is strongly recommended that an endA negative strain of E. coli be used for routine plasmid isolation. Examples of such strains include DH5a® and JM109®.

2. Centrifuge at 10,000 x g for 1 minute at room temperature.

3. Decant or aspirate and discard the culture media.

4. Add 250 µL Solution I/RNase A. Vortex or pipet up and down to mix thoroughly.

Complete resuspension of cell pellet is vital for obtaining good yields.

Note: RNase A must be added to Solution I before use. Please see the instructions in the Preparing Reagents section on Page 6.

5. Transfer suspension into a new 1.5 mL microcentrifuge tube.

6. Add 250 µL Solution II. Invert and gently rotate the tube several times to obtain a clear lysate. A 2-3 minute incubation may be necessary.

Note: Avoid vigorous mixing as this will shear chromosomal DNA and lower plasmid purity. Do not allow the lysis reaction to proceed more than 5 minutes. Store Solution II tightly capped when not in use to avoid acidification from CO₂ in the air.

7. Add 350 µL Solution III. Immediately invert several times until a flocculent white precipitate forms. Note: It is vital that the solution is mixed thoroughly and immediately after the addition of Solution III to avoid localized precipitation.

8. Centrifuge at maximum speed ($\geq 13,000 \times g$) for 10 minutes. A compact white pellet will form. Promptly proceed to the next step.

9. Insert a HiBind® DNA Mini Column into a 2 mL Collection Tube.

10. Add 100 µL Equilibration Buffer.

11. Centrifuge at maximum speed for 30-60 seconds.

12. Discard the filtrate and reuse the collection tube.

13. Transfer the cleared supernatant from Step 8 by CAREFULLY aspirating it into the HiBind® DNA Mini Column. Be careful not to disturb the pellet and that no cellular debris is transferred to the HiBind® DNA Mini Column.

14. Centrifuge at maximum speed for 1 minute.

15. Discard the filtrate and reuse the collection tube.

16. Add 500 µL HB Buffer.

17. Centrifuge at maximum speed for 1 minute.

18. Discard the filtrate and reuse collection tube.

19. Add 700 µL DNA Wash Buffer.

Note: DNA Wash Buffer must be diluted with 100% ethanol prior to use. Please see Page 6 for instructions.

20. Centrifuge at maximum speed for 1 minute.

21. Discard the filtrate and reuse the collection tube.

Optional: Repeat Steps 19-21 for a second DNA Wash Buffer wash step.

22. Centrifuge the empty HiBind® DNA Mini Column for 2 minutes at maximum speed to dry the column matrix.

Note: It is important to dry the HiBind® DNA Mini Column matrix before elution. Residual ethanol may interfere with downstream applications.

23. Transfer the HiBind® DNA Mini Column to a clean 1.5 mL microcentrifuge tube.

24. Add 30-100 µL Elution Buffer or sterile deionized water directly to the center of the column membrane.

Note: The efficiency of eluting DNA from the HiBind® DNA Mini Column is dependent on pH. If using sterile deionized water, make sure that the pH is around 8.5.

25. Incubate at room temperature for 1 minute.

26. Centrifuge at maximum speed for 1 minute. Note: This represents approximately 70% of bound DNA. An optional second elution will yield any residual DNA, though at a lower concentration.

27. Store DNA at -20 °C.

- **E.Z.N.A.® Plasmid Mini Kit Protocol - Low Copy Number Plasmid and BAC DNA Protocol**

1. Increase the volume of starting culture from that of high copy number plasmids. Use 5-10 mL bacterial culture for the E.Z.N.A.® Plasmid DNA Mini Kit I or 20-30 mL bacterial culture for E.Z.N.A.® Plasmid DNA Mini Kit II.

2. Pellet the bacterial cells by centrifugation.

3. Decant or aspirate and discard the culture media.

4. Perform Steps 4-8 in the standard protocols with double the volumes of Solution I, Solution II, and Solution III.

5. Continue with Step 9 of the standard protocols by following the wash, drying, and elution steps. There is no need to increase the volumes of HB Buffer, DNA Wash Buffer, or Elution Buffer.

- **RNeasy Mini Kit: Purification of Total RNA from Animal Tissues**

1-3. Disruption and homogenization of animal tissue

4. Centrifuge the lysate for 3 min at full speed. Carefully remove the supernatant by pipetting, and transfer it to a new microcentrifuge tube (not supplied). Use only this supernatant (lysate) in subsequent steps. In some preparations, very small amounts of insoluble material will be present after the 3 min centrifugation, making the pellet invisible.

5. Add 1 volume of 70% ethanol to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately to step 6.

Note: The volume of lysate may be less than 350 µl or 600 µl due to loss during homogenization and centrifugation in steps 3 and 4.

Note: Precipitates may be visible after addition of ethanol. This does not affect the procedure.

6. Transfer up to 700 µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.

Reuse the collection tube in step 7.

If the sample volume exceeds 700 µl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.

Optional: If performing optional on-column DNase digestion (see “Eliminating genomic DNA contamination”, page 21), follow steps D1–D4 (page 67) after performing this step.

D1. Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the spin column membrane.

Discard the flow-through.

Reuse the collection tube in step D4.

D2. Add 10 µl DNase I stock solution (see above) to 70 µl Buffer RDD. Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube. Buffer RDD is supplied with the RNase-Free DNase Set.

Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex.

D3. Add the DNase I incubation mix (80 µl) directly to the RNeasy spin column membrane, and place on the benchtop (20–30°C) for 15 min.

Note: Be sure to add the DNase I incubation mix directly to the RNeasy spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.

D4. Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.

Continue with the first Buffer RPE wash step in the relevant protocol.

Note: In most of the protocols, the immediately following Buffer RW1 wash step is skipped (as indicated in the protocol). Continue with the first Buffer RPE wash step.

8. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the spin column membrane.

Discard the flow-through.

Reuse the collection tube in step 9.

Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use.

9. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the spin column membrane.

The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

10. Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.

Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 9.

11. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to elute the RNA.

• High Capacity RNA-to-cDNA kit

1. Use up to 2 µg of total RNA per 20 µl reaction.
2. Allow the kit components to thaw on ice
3. Prepare the RT reaction mix on ice:

Component	Volume/Reaction (μL)	
	+RT	–RT
2X RT Buffer	10.0	10.0
20X RT Enzyme Mix	1.0	–
Nuclease-free H ₂ O	Q.S.+ to 20 μL	Q.S. to 20 μL
Sample	up to 9 μL	up to 9 μL
Total per Reaction	20.0	20.0

4. Aliquot 20 μL of RT reaction mix into each well, or tube.
5. Seal the plates or tubes
6. Briefly centrifuge the plate or tubes to spin down the contents and to eliminate air bubbles.
7. Place the plate or tubes on ice until you are ready to load the thermal cycler or Applied Biosystems Real-Time PCR system.
8. Incubate the reaction at 37 °C for 1 hour, stop the reaction by heating to 95 °C for 5 minutes and cool at 4 °C.
9. Long-term storage at -15 to -25 °C

C. Other results:

16S rRNA sequencing:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Piscirickettsia salmonis strain CGA/ID556/2012 16S ribosomal RNA gene, partial sequence	605	605	100%	1e-169	99%	KC473451.1
Piscirickettsia salmonis strain CGA/ID595/2012 16S ribosomal RNA gene, partial sequence	605	605	100%	1e-169	99%	KC473449.1
Piscirickettsia salmonis strain CGA/8417/2012 16S ribosomal RNA gene, partial sequence	605	605	100%	1e-169	99%	KC473442.1
Piscirickettsia salmonis strain CGA/1850/2012 16S ribosomal RNA gene, partial sequence	605	605	100%	1e-169	99%	KC473436.1
Piscirickettsia salmonis strain CGA/1881/2012 16S ribosomal RNA gene, partial sequence	605	605	100%	1e-169	99%	KC473433.1
Piscirickettsia salmonis strain CGA/8998/2012 16S ribosomal RNA gene, partial sequence	605	605	100%	1e-169	99%	KC473432.1
Piscirickettsia salmonis strain CGA/ID495/2012 16S ribosomal RNA gene, partial sequence	605	605	100%	1e-169	99%	KC473430.1
Piscirickettsia salmonis strain CGA/ID432/2012 16S ribosomal RNA gene, partial sequence	605	605	100%	1e-169	99%	KC473426.1
Piscirickettsia salmonis strain CGA/8076/2012 16S ribosomal RNA gene, partial sequence	605	605	100%	1e-169	99%	KC473425.1
Piscirickettsia salmonis isolate SCO-95A 16S ribosomal RNA gene, partial sequence	605	605	100%	1e-169	99%	AY498636.1
Piscirickettsia salmonis isolate SCO-02A 16S ribosomal RNA gene, partial sequence	605	605	100%	1e-169	99%	AY498635.1
Piscirickettsia salmonis 16S ribosomal RNA gene, complete sequence	605	605	100%	1e-169	99%	U55015.1
Piscirickettsia salmonis strain LF-89 16S ribosomal RNA gene, complete sequence >gb U36941.1 PSU36941 Piscirickettsia salmonis	605	605	100%	1e-169	99%	NR_025980.1
Piscirickettsia salmonis strain CGA/ID655/2012 16S ribosomal RNA gene, partial sequence	601	601	100%	1e-168	99%	KC473450.1
Piscirickettsia salmonis strain CGA/8765/2012 16S ribosomal RNA gene, partial sequence	601	601	100%	1e-168	99%	KC473448.1

Fig. 3.1: Sequences alignments for *P. salmonis* 5892 in the BLAST program.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Piscirickettsia salmonis strain CGA/ID556/2012 16S ribosomal RNA gene, partial sequence	165	165	100%	5e-38	100%	KC473451.1
Piscirickettsia salmonis strain CGA/ID655/2012 16S ribosomal RNA gene, partial sequence	165	165	100%	5e-38	100%	KC473450.1
Piscirickettsia salmonis strain CGA/ID595/2012 16S ribosomal RNA gene, partial sequence	165	165	100%	5e-38	100%	KC473449.1
Piscirickettsia salmonis strain CGA/8765/2012 16S ribosomal RNA gene, partial sequence	165	165	100%	5e-38	100%	KC473448.1
Piscirickettsia salmonis strain CGA/8686/2012 16S ribosomal RNA gene, partial sequence	165	165	100%	5e-38	100%	KC473447.1
Piscirickettsia salmonis strain CGA/8588/2012 16S ribosomal RNA gene, partial sequence	165	165	100%	5e-38	100%	KC473445.1
Piscirickettsia salmonis strain CGA/8465/2012 16S ribosomal RNA gene, partial sequence	165	165	100%	5e-38	100%	KC473444.1
Piscirickettsia salmonis strain CGA/8423/2012 16S ribosomal RNA gene, partial sequence	165	165	100%	5e-38	100%	KC473443.1
Piscirickettsia salmonis strain CGA/8417/2012 16S ribosomal RNA gene, partial sequence	165	165	100%	5e-38	100%	KC473442.1
Piscirickettsia salmonis strain CGA/8383/2012 16S ribosomal RNA gene, partial sequence	165	165	100%	5e-38	100%	KC473441.1
Piscirickettsia salmonis strain CGA/8379/2012 16S ribosomal RNA gene, partial sequence	165	165	100%	5e-38	100%	KC473440.1
Piscirickettsia salmonis strain CGA/8376/2012 16S ribosomal RNA gene, partial sequence	165	165	100%	5e-38	100%	KC473439.1
Piscirickettsia salmonis strain CGA/8337/2012 16S ribosomal RNA gene, partial sequence	165	165	100%	5e-38	100%	KC473438.1
Piscirickettsia salmonis strain CGA/8079/2012 16S ribosomal RNA gene, partial sequence	165	165	100%	5e-38	100%	KC473437.1
Piscirickettsia salmonis strain CGA/1850/2012 16S ribosomal RNA gene, partial sequence	165	165	100%	5e-38	100%	KC473436.1

Fig. 3.2: Sequences alignments for *P. salmonis* 5896 in the BLAST program.

Zebrafish embryo immune response to *P. salmonis* 5692 infection:

A: Ct-values from Tg(LysC:DsRED2) embryos infected day=1

	18S	EF1a	TNFa	ilB	il8	il10	IFNy	Socs3b
5692 calcein	13,91	21,12	38,19	30,21	26,63	33,2	34,6	24,65
	14,08	21,69	37,28	30,97	26,07	34,11	35,71	25,13
	14,51	21,69	40	31,02	27,62	35,97	35,93	26,6
	13,99	20,46	38,35	31,25	27,08	36,18	36,55	26,1
PBS	14,53	22,82	40	29,6	28,01	34,78	36,56	26,26
	14,2	22,3	38,25	29,61	27,98	34,34	38,39	26,44
	13,77	22,34	40	30,04	26,15	33,72	35,07	25,74
	13,82	22,42	40	30,54	26,23	33,81	36,03	25,77
	14,83	23,18	37,02	26,87	24,87	33,44	36,49	25,55
	14,85	23,32	37,43	26,77	24,94	34,09	36,04	25,51

B: Normalized to PBS values from Tg(LysC:DsRED2) embryos infected day=1

	18S	EF1a	TNFa	ilB	il8	il10	IFNy	Socs3b
5692 calcein	0,66163826	1,50601539	0,41578053	0,08639976	0,24350803	0,800671	1,44195263	1,04580559
5692 calcein	0,76002278	1,31106255	1,00967718	0,06593422	0,46395001	0,55067822	0,86335378	0,96903097
5692 calcein	0,65479471	1,52175543	0,17786988	0,07392321	0,1839061	0,17607738	0,86036681	0,40601774
5692 calcein	0,51196411	1,94630326	0,30436916	0,03436708	0,14579784	0,08300151	0,30524166	0,31308248
PBS	0,96200385	1,03579358	0,26496875	0,29466981	0,20906758	0,59844122	0,82818431	0,7655745
PBS	0,9006975	1,10629529	0,66383385	0,21796542	0,15899367	0,6046958	0,17350195	0,50334332
PBS	1,06003696	0,94000251	0,17240746	0,14133309	0,49381455	0,81184795	1,51364173	0,71430627
PBS	1,07111588	0,93027974	0,18035285	0,1045432	0,48870686	0,79790089	0,81395659	0,73184736
PBS	0,98221759	1,01447725	2,62787036	2,45746547	2,31665187	1,9043273	1,09279574	1,57419546
PBS	1,02392821	0,97315163	2,09056674	2,78402303	2,33276546	1,28278684	1,57791968	1,71073309

C: Ct-values from Tg(LysC:DsRED2) embryos infected day=6

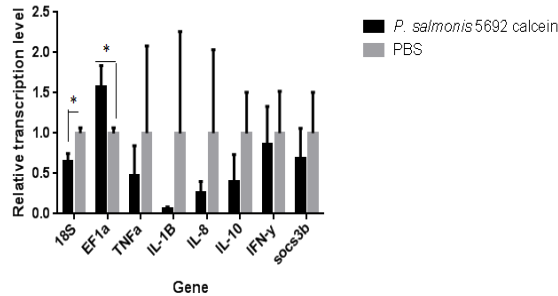
	18S	EF1a	TNFa	ilB	il8	il10	IFNy	Socs3b
5692 calcein	13,55	19,72	40	27,04	25,92	33,67	34,2	24
	13,73	19,22	37,48	26,04	25,67	33,44	32,85	23,55
	14,82	21,11	40	26,92	27,62	34,96	35,14	25,73
	14,21	21,59	37,95	26,83	27,08	35,25	34,34	25,58
PBS	13,85	21,26	38,08	26,13	25,5	33,64	33,99	24,32
	14,15	20,86	38,1	25,61	24,66	32,9	33	24
	13,93	20,13	38,41	25,49	25,17	33,17	33,17	24,01
	14,24	19,8	37,43	25,68	25,19	32,83	33,57	24,12
	14,11	19,91	36,62	25	24,1	33,2	33,45	23,67

D: Normalized to PBS values from Tg(LysC:DsRED2) embryos infected day=6

	18S	EF1a	TNFa	ilB	il8	il10	IFNy	Socs3b
5692 calcein	0,91874175	1,03265615	0,12756639	0,23791998	0,31515923	0,45281815	0,37666152	0,66835806
5692 calcein	0,72584394	1,30709133	0,65488803	0,42588869	0,33544609	0,47533077	0,8593636	0,81716149
5692 calcein	0,95775682	0,99058999	0,32070531	0,65001653	0,2438647	0,46554861	0,49357478	0,50652069
5692 calcein	1,39738668	0,67894187	1,26955259	0,66137862	0,33895199	0,36399832	0,82150461	0,53726029
PBS	1,41199139	0,67191934	0,91340093	0,84589372	0,79782976	0,87478701	0,82435667	1,01304139
PBS	1,10782599	0,85640193	0,87014043	1,1716554	1,37950706	1,41127947	1,58155155	1,22153323
PBS	0,92834393	1,02197503	0,5049877	0,91608179	0,69696245	0,84206512	1,01139081	0,87278192
PBS	0,74366836	1,27576266	0,98919041	0,79749552	0,68261917	1,05848711	0,76119636	0,80312314
PBS	0,80817033	1,17394104	1,72228054	1,26887356	1,44308156	0,81338129	0,82150461	1,08952033

E.

Immune response of LysC:DsRED2 embryos infected with Calcein-AM stained *P. salmonis* 5692- day 1



F.

Immune response of LysC:DsRED2 embryos infected with Calcein-AM stained *P. salmonis* 5692- day 6

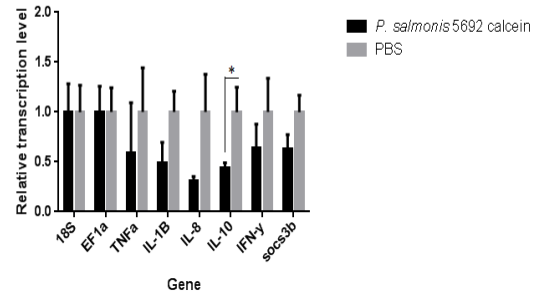


Fig. 3.3: Immune response of Tg(LysC:DsRED2) embryos infected with *P. salmonis* 5692 at different time points (*= significant).

A: Ct-values from Tg(mpeg1:gal4ff):(UAS:nfsB-mCherry) embryos infected day=1

	18S	EF1a	TNFa	ilB	il8	il10	IFN γ	Socs3b
5692 calcein	14,51	22,9	35,26	31,53	27,49	33,61	36,26	26,13
	13,51	24,12	34,45	31,44	28,11	36,09	40	26,95
	13,07	20,49	35,1	30,67	26,22	33,65	35,61	25,29
	13,27	22,84	36,21	30,63	25,67	32,66	34,68	24,74
	13,73	21,65	40	30,94	27,53	34,12	34,74	25,18
	12,7	21,43	36,52	31,21	26,79	33,61	35,86	24,8
5692+calcein	14,04	30,46	37,72	32,54	28,73	34,67	36,94	27,29
	12,99	21,48	35,8	31,7	28	36,56	40	27
	14,73	21,82	35,23	31,51	28,09	35,43	37,62	26,97
PBS	14,6	21,83	38,42	31,43	26,69	34,59	36,69	26,86
	14,2	21,59	40	31,76	26,83	36,6	36,29	26,93
	14,51	20,78	35,02	30,26	27,66	35,82	36,72	25,9
	14,12	20,87	33,82	30,77	27,78	34,71	38,19	25,59
	14,2	21,23	37	28,84	26,2	34,96	36,44	26,44
	14,79	20,46	36,15	28,87	26,57	35,57	36,61	26,71

B: Normalized to PBS values from Tg(mpeg1:gal4ff):(UAS:nfsB-mCherry) embryos infected day=1

	18S	EF1a	TNFa	ilB	il8	il10	IFN γ	Socs3b
5692 calcein	1,74627136	0,54902863	2,67110626	0,63987058	1,1888254	5,67540366	2,53717958	2,24678512
5692 calcein	3,76925071	0,25436169	5,054041	0,73501828	0,83481987	1,097882	0,2049332	1,37350499
5692 calcein	1,24770577	0,76841271	0,78591074	0,30584064	0,75499388	1,45369338	1,04842644	1,05911713
5692 calcein	2,62856928	0,36474327	0,88113646	0,76093865	2,67499621	6,9872415	4,83402736	3,75252622
5692 calcein	1,48378058	0,64615549	0,04946107	0,47660039	0,57217836	1,97208674	3,6005708	2,14780372
5692 calcein	1,96465741	0,48800008	0,3578526	0,25629052	0,61965489	1,82099001	1,07417246	1,8123507
5692+calcein	28,2323587	0,03395936	5,66641933	3,70854182	5,87478823	31,772859	18,4842633	11,7359454
5692+calcein	1,80785348	0,5303267	0,6631639	0,20530662	0,3013552	0,26512103	0,06854644	0,44376327
5692+calcein	1,11286436	0,86151826	2,02432	0,48158155	0,58217986	1,19310665	0,73368141	0,93165141
PBS	1,16819228	0,82071503	0,21278043	0,48830418	1,473796	2,04872026	1,3409223	0,96450602
PBS	1,23480032	0,77644373	0,0570131	0,31118664	1,07142944	0,40745793	1,41737906	0,7360431
PBS	0,83756773	1,14468709	1,51302662	0,74013074	0,50681701	0,5883404	0,88467879	1,26388233
PBS	0,98916134	0,96925843	3,13276677	0,46841268	0,42031325	1,1445044	0,28781738	1,41211947
PBS	1,08996194	0,87962059	0,4026051	2,07894565	1,46361575	1,12095084	1,12757675	0,9124783
PBS	0,68031639	1,40927512	0,68180797	1,91302011	1,06402854	0,69002618	0,94162572	0,71097077

C: Ct-values from Tg(mpeg1:gal4ff):(UAS:nfsB-mCherry) embryos infected day=6

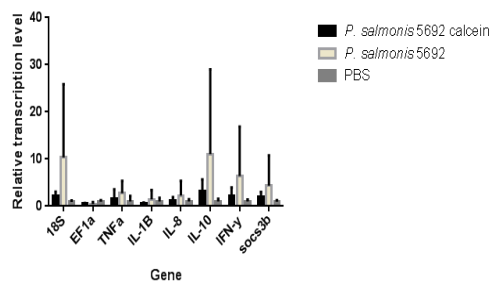
	18S	EF1a	TNFa	ilB	il8	il10	IFNy	Socs3b
5692 calcein	19,53	26,54	32,83	26,96	26,1	32,76	34,02	26,61
	17,85	25,48	32,87	26,61	26,29	33,58	33,54	25,55
	13,98	20,08	34,94	26,34	26,29	32,95	32,26	23,62
	14,18	19,63	35,58	26,33	26,17	32,16	32,48	23,68
	14,67	20,67	35,06	26,08	25,9	32,91	40	24,02
	14,72	24,41	34,63	26,23	25,7	34,9	34,65	23,82
5692+calcein	14,06	23,8	31,26	25,75	24,8	33,92	33,67	24,11
	13,74	22,5	31,52	25,33	25,05	32,54	33,58	23,65
	14,6	22,33	34,2	28,13	27	34,34	35,06	23,68
	14,56	21,65	33,11	27,59	26,24	35,55	33,77	24,43
PBS	14	21,63	34,72	25,2	25,07	33,24	34,57	24,3
	14,98	21,81	34,52	25,18	24,73	34	33,68	24,24

D: Normalized to PBS values from Tg(mpeg1:gal4ff):(UAS:nfsB-mCherry) embryos infected day=6

	18S	EF1a	TNFa	ilB	il8	il10	IFNy	Socs3b
5692 calcein	0,92658806	1,07922824	105,41965	8,9382971	13,2691127	55,3303828	32,7858183	6,02098699
5692 calcein	1,14869835	0,87055056	39,6706464	4,40762046	4,50023394	12,1257325	17,6916905	4,85677954
5692 calcein	0,67595542	1,47938751	0,38024469	0,21389876	0,18111777	0,75523629	1,72907446	0,74483873
5692 calcein	0,53961412	1,85317612	0,22375627	0,19751033	0,18049115	1,1974787	1,36131412	0,6551967
5692 calcein	0,65292989	1,531558	0,54525387	0,39914919	0,36984688	1,20999409	0,01260378	0,87964908
5692 calcein	2,3456699	0,42631745	2,73208051	1,33792755	1,58008262	1,13288389	1,91189064	3,758091
5692+calcein	2,38667149	0,41899357	18,1890716	1,20163605	1,89868424	1,43893358	2,42838977	1,97931331
5692+calcein	1,69937	0,58845337	8,66380036	0,91700404	0,91066983	2,13613082	1,47426922	1,55293775
5692+calcein	1,18920712	0,84089642	1,71713087	0,16724094	0,29936968	0,77916458	0,67128625	1,93187266
5692+calcein	0,952638	1,04971668	2,84810039	0,18946457	0,39502066	0,26242917	1,27898558	0,89502507
PBS	1,14869835	0,87055056	0,7631296	0,8122524	0,72698626	1,06437018	0,60081802	0,80106988
PBS	0,87055056	1,14869835	1,3103934	1,23114441	1,37554182	0,93952275	1,66439747	1,24833055

E.

Immune response of Mpeg1:gal4ff:UAS:nfsB-mCherry embryos infected with Calcein-AM stained and non-stained *P. salmonis* 5692- day 1



F.

Immune response of Mpeg1:gal4ff:UAS:nfsB-mCherry embryos infected with Calcein-AM stained and non-stained *P. salmonis* 5692- day 6

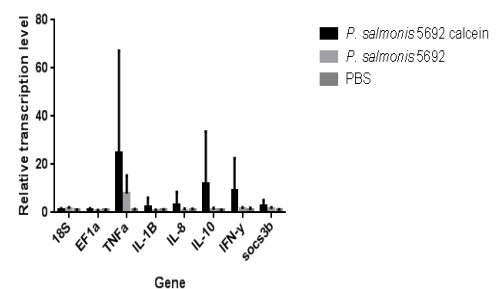


Fig. 3.4: Immune response of Tg(mpeg1:gal4ff):(UAS:nfsB-mCherry) embryos infected with *P. salmonis* 5692 at different time points (*= significant).